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AUG 14 1942

VOL. XXIII. No. 3] [JUNE, 1942

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

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LONDON
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136 GOWER ST., W.C. 1

CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO

on behalf of

'The British Journal of Experimental Pathology.' Registered Office: 120 Moorgate, London, E.C. 2

Annual Subscription, January to December, post free, Two Pounds net
Single Numbers, Seven and Sixpence net

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

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Subscriptions and business correspondence are received by the publishers, Messrs. H. K. Lewis & Co. Ltd., 136, Gower Street, W.C. 1.

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THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XXIII JUNE, 1942 No. 3

PURIFICATION AND SOME PHYSICAL AND CHEMICAL PROPERTIES OF PENICILLIN.

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From the Sir William Dunn School of Pathology, Oxford.

WITH A NOTE ON

THE SPECTROGRAPHIC EXAMINATION OF PENICILLIN PREPARATIONS.

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Received for publication March 27, 1942.

Chemotherapeutic and other properties of partially purified penicillin have been described by Chain *et al.* (1940) and by Abraham *et al.* (1941). In the present communication a method of obtaining a highly purified penicillin preparation and a description of some of its physical and chemical properties are given.

EXPERIMENTAL.

Assay Method.

The ring method previously described (Abraham *et al.*, 1941) has been employed throughout for assaying the antibacterial power of penicillin preparations. Occasionally the values obtained by the ring method were checked by the serial dilution method. The relationship between the ring and the serial dilution methods is given in the following paper by Florey and Jennings (1942).

Production of Crude Product.

The type of vessel used for growing the mould, the composition and quantities of the medium and the temperature of incubation have been previously described (Abraham *et al.*, 1941). Batches of 50 vessels, each vessel containing 1 l. of medium, were set up daily. They were autoclaved at 1 atm. for

* With personal grants from the Medical Research Council.

1 hour, and inoculated on the next day with a spore suspension derived from subcultures of a strain of Penicillium originally obtained from Prof. A. Fleming. The spore suspension was made by adding sterile water, from a hooded pipette, to a slope culture on Sabouraud's medium and shaking vigorously. A Pasteur pipette was used to inoculate the vessels. The greatest care was taken to avoid contamination of the spore suspension, as even very small numbers of certain air-borne bacteria growing in the vessels together with the mould can reduce the penicillin yield to vanishing point. After the mycelium had fully covered the surface of the medium and the pH had reached 7·0–7·4 (usually after 10 days) the liquid (containing the penicillin) was drawn off and the mycelium discarded. The replacement of the exhausted medium by fresh (as previously described by Abraham *et al.* (1941)) was found frequently to result in contamination and was consequently discontinued.

The usual yield of penicillin was about 4 units per c.c. of culture fluid. Occasionally higher values (6–8 units) and rarely much lower values were obtained. In a few exceptional batches no penicillin at all was produced. The reasons for these inconsistencies, which have also been observed in other laboratories, are not yet fully understood, but possibly contamination of the inoculum was responsible for the few very poor yields.

Purification of Penicillin.

Distribution between solvents, absorption of impurities by charcoal and chromatographic separation.

The culture fluid was chilled in the refrigerator to 4° C., acidified with 10 per cent. phosphoric acid to pH 1·9 and extracted in batches of 2 l. with an equal volume of amyl acetate. The latter removed 90–95 per cent. of the active material. There was evidence from activity and absorption spectra measurements (E. R. Holiday, see below) that more impurities were extracted by this method than by the counter-current apparatus. However, the direct extraction was introduced because it was found that even when the column of the counter-current extraction apparatus was lengthened to 14 ft. only 80 per cent. of the penicillin was extracted. With present resources it was not possible to construct a longer column, though the latter would obviously be more suitable for large-scale extraction of penicillin. The penicillin was extracted from the amyl acetate solution into water by shaking 2 l. lots with 400 c.c. water, $N/30$ baryta being added in small portions with vigorous shaking to bring to pH 6·2–6·4. The temperature during these procedures did not exceed 7° C. It was usually found that 30–40 c.c. $N/30$ baryta was necessary for each 2 l. batch, and fresh water was used only after ten 2 l. batches of amyl acetate had been extracted. Nearly all the penicillin activity was recovered by this method. The residual amyl acetate was again washed with water (without further addition of baryta) to remove remaining parts of the first strong extract. The distribution of penicillin and impurities between amyl acetate and water at pH 5·5–6·0 (Fig. 1) is such that a considerable purification is effected. The first extract obtained from 260 l. amyl acetate was approximately 8 l. of a strongly pigmented red-brown solution. This was filtered and treated with B.D.H. animal charcoal (40–60 g./l.). Very little

penicillin activity was lost in this process if the correct amount of charcoal was used, but a great deal of inactive pigment was removed which obscured the zones of the chromatogram on the alumina column used in the subsequent purification process. It was advisable to determine for each batch, on a small sample, the amount of charcoal necessary to remove the maximum of pigment without losing penicillin activity. The solution was filtered, the charcoal residue washed and the washings added to the solution. The latter

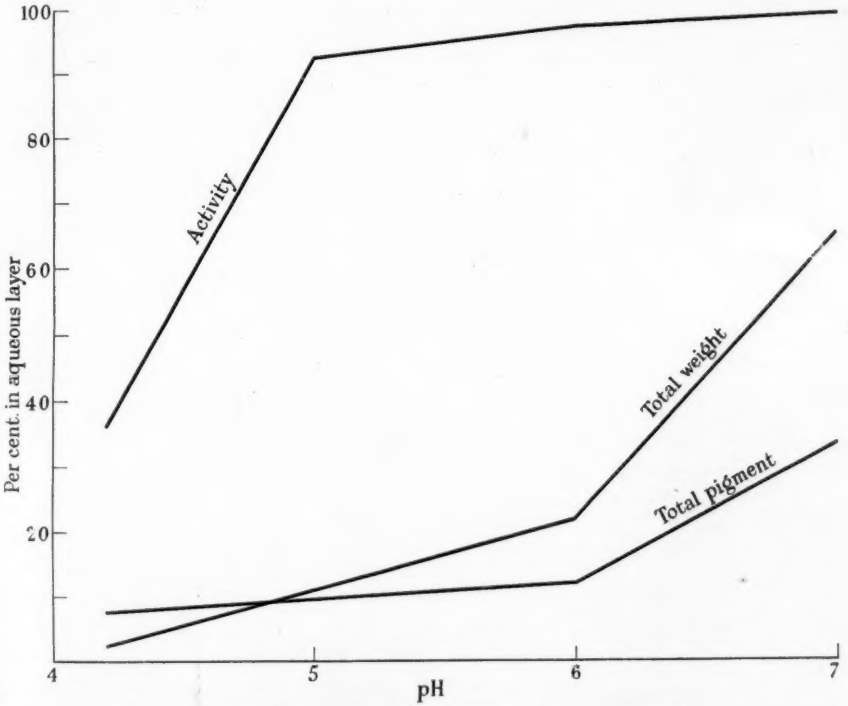


FIG. 1.—Distribution of crude material between water and amyl acetate.

was chilled to 4° C., and one-third of its volume of cold ether (B.D.H. "Anaesthetic") added. The watery layer was acidified to pH 1·9 with 10 per cent. phosphoric acid and extracted by vigorous shaking. Three extractions with ether were made in this way and the temperature was not allowed to rise above 4° C. (Solid carbon dioxide was used for cooling the ether and water solutions.)

Since purified penicillin is destroyed by peroxides, these were removed (Brandt, 1927) from ether used in subsequent stages of purification.

The ether solution of penicillin (containing about one million units) was then passed through an alumina column 40 cm. long and 3·6 cm. diameter.

The alumina used (standardized according to Brockmann) was obtained from Messrs. Savory & Moore, London.

The following chromatogram was obtained:

(1) A dark brown layer, 1·0 cm. long, containing some penicillin.

(2) A light yellow layer, 12–14 cm. long, containing the bulk of the penicillin.

(3) An orange-yellow (or, in some batches, lemon-yellow) layer of varying length, containing small amounts of penicillin.

(4) A purple layer containing no penicillin.

A further yellow pigment was unadsorbed and passed through the column. Only fraction (2) was used for further purification. This was separated mechanically from the other fractions on the column and the alumina was shaken with $M/15$ phosphate buffer (pH 7), which eluted the penicillin. Four elutions were carried out, the first with 600 c.c. of phosphate for 30 minutes, the second with the same volume for 1 hour, the third with the same volume for 2 hours, and the fourth with the same volume for 4 hours.

The combined (yellow-coloured) eluates were extracted at pH 2 three times with one-third of their volume of peroxide-free ether at 4° C. From the ether solution the penicillin was brought back into water (one-fifth of its volume) by adjusting to pH 5·8–6·0 with $N/30$ baryta. Inactive pigment remained in the ether layer. The active substance was then again extracted by ether in the usual manner and the ether solution passed through an alumina column 25 cm. long and 2·5 cm. wide. The chromatogram again consisted of 4 layers, but fractions (1), (3) and (4) were proportionately small in comparison with the corresponding fractions in the first chromatogram. The largest and most active layer (fraction (2)) was about 12 cm. long. After extraction of this with phosphate as above the penicillin was transferred to ether as previously described and then extracted with $N/30$ baryta at pH 5·8–6·0. Again inactive pigment remained in the ether layer. The barium salt of penicillin in this state of purity was a light yellow powder. If there was doubt as to the points at which the chromatogram should be divided, the column was cut into lengths of 1 cm. and 10 mg. of each portion eluted twice with 1 c.c. of phosphate buffer: the penicillin content of each sample was assayed.

Reduction by aluminium-mercury couple.

The next step in the purification process was reduction with aluminium amalgam. The latter was prepared from aluminium turnings about 2 mm. square. The pieces were treated with 5 per cent. soda until evolution of hydrogen became vigorous, washed exhaustively with tap water, and amalgamated by shaking vigorously for two minutes with 0·5 per cent. mercuric chloride in water. They were then washed again with water, alcohol and petroleum ether and stored under petroleum ether until required. The reduction was carried out as follows: 1 g. of the barium salt of penicillin, purified as described above, was dissolved in 150 c.c. of water. To the solution were added 1 g. of aluminium-mercury couple and 15–20 c.c. of ether to prevent frothing. As the reduction proceeded, hydroxyl ions were set free, rapidly at the beginning, more slowly towards the end of the reduction. The pH was

continuously readjusted to 7·0 by adding $N/10$ HCl. Normally, approximately 20 c.c. of acid were used for 1 g. of barium salt of penicillin. The yellow pigment was precipitated together with aluminium hydroxide and the suspension gradually became green. On centrifuging during the course of the reduction it was found that the supernatant solution became less coloured, and the reduction was interrupted when the production of hydroxyl ions became very slow and supernatant liquid had become nearly colourless. The time necessary for the completion of the reduction depended on the activity of the aluminium-mercury couple used ; it should not exceed four hours. An assay of the penicillin activity of the supernatant liquid showed that only 10–25 per cent. of the activity had been lost. After centrifuging and filtering, the solution was cooled to 4° C. and extracted twice with one-half of its volume of cold amyl acetate. From the filtered amyl acetate solution penicillin was extracted into one-fifth of its volume of water, the pH being adjusted to 7·0 with $N/30$ baryta. The activity of the material at this stage was approximately 300 units per mg.

Further chromatographic adsorption.

A solution of the barium salt (10 mg. per c.c.) was extracted with amyl acetate at pH 2 and 5° C. and the extract passed through an alumina column. Three fractions were obtained : (1) very light brown, (2) almost colourless, (3) greenish yellow. The lengths of the column these fractions occupied were in the ratio 1 : 12 : 1·7, and the activities of the material associated with them were in the ratio 1 : 1·5 : 1. Division of the column was facilitated by observation in ultra-violet light, fractions (1) and (3) being fluorescent. The penicillin was eluted from fraction (2) with phosphate buffer. After two further chromatographic adsorptions from amyl acetate the resulting column was almost colourless and homogeneous. Elution with phosphate, extraction into amyl acetate and extraction with $N/30$ baryta at pH 6·5 yielded a solution from which the barium salt was obtained as a very faintly yellow powder on lyophylic drying. Its activity was 450–500 units per mg.

The degree of purification achieved in the various stages of the purification process is shown in Table I.

TABLE I.—*Activity of Penicillin (Barium Salt) at Different Stages of the Purification Process.*

	Units /mg.	Recovery (per cent.).
Material in original culture fluid . . .	0·3–0·5	..
After amyl acetate extraction . . .	15–25	96
After first alumina column	70–100	60–80
After second alumina column . . .	100–150	50–60
After reduction with aluminium amalgam .	300	30
After fifth alumina column	450–500	15

The course of purification was also followed by measurements of absorption spectra (see following note). The figures in Table I represent the normal course of purification of penicillin. During the working-up of a large number

of batches we have obtained fairly constant figures, but in occasional batches, which may have contained appreciable amounts of inactivated penicillin, the purification was not nearly so effective. It appears to be essential to start from culture medium in which no enzymatic inactivation of penicillin has occurred if a final product with a high antibacterial activity is to be obtained.

Analysis.

The C and H content of a number of preparations at various stages of purification showed no great variation. The N content, however, increased with the activity. The following analytical figures (Weiler) are for the most active barium salt after drying at 100° C. in vacuo (6·5 per cent. loss in weight): C, 44·3; H, 4·85; N, 4·13; Ba, 22·0; C–Me, 11·6 per cent. P, Halogen, S and OMe were absent. An independent determination of the Ba content gave 21·3 per cent., and the N content as determined by Conway's modification of the Kjeldahl method was 4·2 per cent.

Determination of acetylatable groups was carried out by the method of Freed and Winne (1936). Titration of the excess of acetic anhydride gave an apparent value of one group per Ba atom. Since, however, a new titratable group would probably have been formed from the penicillin under the conditions of the reaction (*vide infra*), the true figure is likely to be two groups per barium atom.

On the assumption that this material is practically pure its barium content corresponds to a molecular weight (calculating for a dibasic acid) of approximately 640. From the elementary analysis, formulae such as $C_{24}H_{32}O_{10}N_2Ba$ (mol. wt. 645) and $C_{23}H_{30}O_9N_2Ba$ are possible (*cf.* Abraham *et al.*, 1942).

Solubility of Penicillin and Its Salts.

Free penicillin is very soluble in ether, alcohol, acetone, ethyl acetate, amyl acetate, cyclohexanon and dioxan; it is less soluble in benzene, chloroform and carbon tetrachloride. It is hygroscopic and rapidly loses activity if exposed to the air, presumably by hydrogen ion inactivation. If absolutely dry it retains its activity for at least 24 hours. It can be obtained as an easily soluble powder by drying from the frozen state in a high vacuum, either from water or dioxane solution.

The sodium, potassium and ammonium salts of penicillin are very hygroscopic. The barium salt is a non-hygroscopic white powder and is the most suitable salt for general chemical use. When the following cations were added to a solution of the sodium salt (10 mg./c.c.) no precipitate, or only a very small one, was formed: Calcium, strontium, all rare earths, zinc, cadmium, mercury, copper, lead, uranium, nickel, silver, strychnine, brucine, cocaine, morphine, quinine, cinchonine, cinchonidine, acridine, proflavine, Nile blue, malachite green. Ferric iron was the only heavy metal cation found to give a precipitate. In more concentrated solution the alkaloids quinine, cinchonine and cinchonidine, as well as some of the dyes, gave amorphous precipitates when an excess of the precipitant was added. However, these derivatives were not found to be of value for purification purposes. The alkali and alkaline earth salts of pencillin showed the peculiarity of being readily soluble

in absolute methyl alcohol, but they were quite insoluble in absolute ethyl alcohol at room temperature.

Stability of Penicillin.

Effect of temperature and pH on biological activity.

Penicillin in the form of the dried barium salt keeps its anti-bacterial power undiminished for an indefinite period. In aqueous solution it is most stable between pH 5·5 and 7·5, retaining its activity at 2° C. for several months, at 25° C. for several weeks, and at 37° C. for 24 hours ; at 100° C. most of the activity is lost in 30 minutes. Table II shows the stability at different pH values.

TABLE II.—*Stability of the Barium Salt of Penicillin in Acid and Alkali.*

Penicillin dissolved in—	Loss of activity (per cent.) in 3 min. at— 25° C.	8° C.
0·2 *N* HCl	over 80	..
0·1 *N* HCl	approx. 50	0
0·2 *N* NaOH	over 95	30
0·1 *N* NaOH	over 70	0
0·05 *N* NaOH	approx. 30	0

pH of solution.	Temperature (° C.).	Time for which activity retained without detectable loss (min.).
2·0	0	At least 60
2·0	25	5
8·0	37	180
9·0	37	180 (40 per cent. activity lost)

Penicillin dissolved as the free acid in ether or amyl acetate is stable for several days at room temperature, but is inactivated by passing dry HCl or HBr through the solutions.

Acid-base properties. Mechanism of acid, alkali and heat inactivation.

The purest penicillin preparation was titrated electrometrically in order to obtain information about ionizable groups and the effect on the latter of the acid and alkaline treatment which results in biological inactivation. E. R. Holiday (see following note) found that acid or alkaline inactivation produced no change in the ultra-violet absorption spectrum.

Titrations were carried out, at 25° C., in a cell containing a hydrogen electrode and connected by an agar-potassium chloride bridge to a saturated potassium chloride-calomel electrode. The apparatus was standardized with 0·1 *N* HCl, the pH of which was taken as 1·09.

The curves (Figs. 2 and 3) show equivalents of hydrogen or hydroxyl ions bound, per atom of barium, at various pH values. The " blank corrections " necessary for the calculations were obtained experimentally. Owing to the

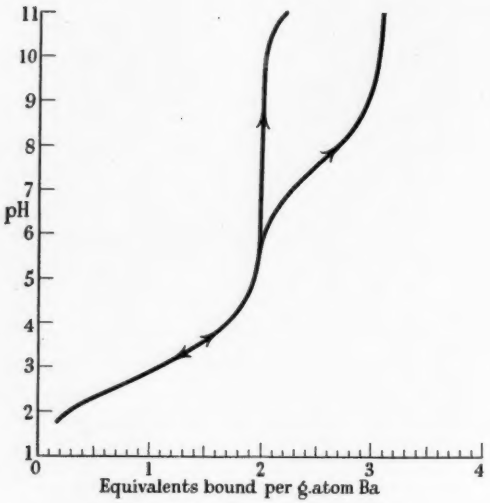


FIG. 2.—Acid titration of penicillin.

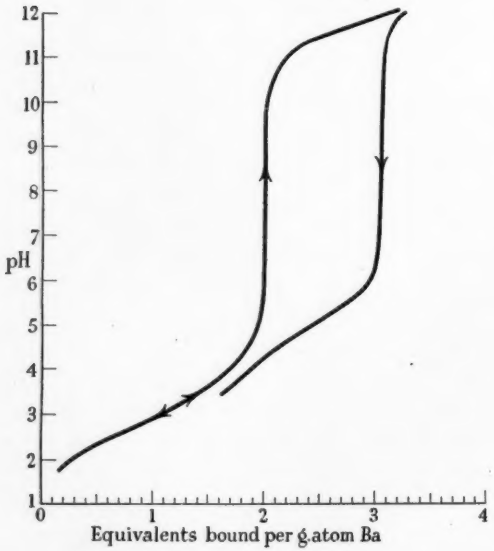


FIG. 3.—Alkaline titration of penicillin.

possible errors in the blank corrections, titrations were not carried much below pH 2 or above pH 12, although at neither of these points were they complete.

Approximate pK values were determined from the half-titration points. A more accurate determination was not considered worth while at this stage.

About 6 mg. of barium salt, in 1 c.c. water, was used for each titration.

(1) *Acid titration* (Fig. 2).—Between pH 6 and pH 2 the most active penicillin preparation behaved as a strong dibasic acid whose dissociation constants were not sufficiently different to produce an obvious inflection in the titration curve. The titration constants of the acidic groups were approximately 2·3 and 3·5. In the range pH 6 to pH 2 the titration was reversible, but by carrying the back-titration beyond pH 6 it was shown that a new group, with a titration constant of approximately pH 7·6, was formed during the acid treatment. The curve showing this was obtained by beginning the reverse titration after the penicillin had been completely inactivated by standing for 30 minutes at pH 1·64. The corresponding curve for active penicillin, obtained by titrating with alkali from the neutral point, showed that this had no buffering power between pH 6 and pH 10.

(2) *Alkaline titration* (Fig. 3).—At pH 11 buffering began sharply, and between pH 11 and pH 11·7 the potentials showed a tendency to fall which suggested that a reaction was occurring. Readings were taken about one minute after each addition of alkali. The back titration was begun after the penicillin had been completely inactivated by standing for 15 minutes at pH 12·2. The curve showed that a reaction had occurred above pH 11, resulting in the formation of a new group with a titration constant of approximately 5·0.

(3) *Effect of alkali after acid inactivation, and of acid after alkaline inactivation.*—The alkaline titration (pH 6 to pH 12·2) of penicillin which had been inactivated by acid was reversible. After the production, in acid, of the new group of pK 7·6, the further breakdown in alkali, between pH 11 and pH 12, did not occur. Similarly, after production of the group of pK 5·0 by alkaline inactivation, the group of pK 7·6 did not appear after further treatment with acid.

(4) *Effect of heat.*—All penicillin preparations lost CO_2 on heating at 100° C. for 1–2 hours in acid, neutral or alkaline solution. This increased in amount with purification, and with the purest preparation the loss of CO_2 corresponded to one molecule per atom of Ba.

The titration curve of the barium salt after boiling for 2 hours in water gave no indication that the CO_2 evolved was derived from one of the acidic groups present originally. There was more buffering between pH 6 and pH 2 and between pH 6 and pH 11 than with active penicillin ; the increase corresponded in all to nearly one group per atom of barium. The titration was completely reversible, but the curve was less clear-cut than those obtained after acid and alkaline inactivation and showed characteristics of the curves obtained after both these processes.

Inactivation by metal cations.

While trying to prepare salts of penicillin it was noticed that the substance

was inactivated by a large number of metal cations. The metals with the most powerful inactivating effect were copper, lead, zinc and cadmium ; but other heavy metals, such as nickel, mercury and uranium, also exerted an inactivating effect. The mechanism of this inactivation has not yet been investigated. It was a time reaction and occurred under anaërobic conditions. Probably complex-formation took place between the metals and the penicillin molecule. No activity could be recovered by decomposing the inactivated material with acid and extracting with ether.

Inactivation by primary alcohols.

During attempts to crystallize the Ba salt of penicillin from warm ethyl alcohol it appeared that activity was being lost. This was confirmed in subsequent experiments with other primary alcohols.

In methyl alcohol the barium salt was stable for 3 hours at room temperature, but at 37° C. 80 per cent. of the activity was lost, and at 50° C. inactivation was complete. Control solutions in water lost no significant amount of activity. (No $—OCH_3$ was found in the inactivated material after evaporating the solution to dryness in a desiccator.)

The Ba salt was not soluble in ethyl alcohol at room temperature, but at 50° C. about 50 per cent. of the activity was lost in 3 hours.

In benzyl alcohol, in which the Ba salt was easily soluble, 90 per cent. of the activity was lost at 37° C. in 15 hours, and in ethylene glycol (under the same conditions) inactivation was complete.

Attempts were made to reactivate penicillin inactivated by methyl alcohol by standing in water at 37° C. and at room temperature. These were unsuccessful.

Inactivation of penicillin by bases.

Ammonia, aniline and quinine in the ionized state had no inactivating effect on penicillin.

When, however, dry ammonia was passed through a dry etheral solution of free penicillin a precipitate was formed possessing no antibacterial properties. Partial inactivation of penicillin also resulted when free aniline or quinine was added to a solution of penicillin in dry ether.

Effect of oxidizing and reducing agents.

In aqueous solution penicillin was stable towards atmospheric oxygen between pH 5–7. Purified penicillin did not reduce Fehling's solution with the formation of cuprous oxide, either in the cold or in the boiling water bath ; but the blue colour of the solution changed during the reaction to a green colour, probably because of complex formation. It was, however, easily oxidized, with loss of activity, by a number of oxidizing agents, such as hydrogen peroxide and permanganate.

Penicillin was oxidized by alkaline ferricyanide, and titration of the ferrocyanide formed (Miller and van Slyke, 1936) showed the presence of four reducing equivalents per Ba atom. After boiling for 30 minutes in *N* HCl or in neutral solution the reducing power fell to approximately half this value.

Hydrogenation experiments were carried out in the Warburg apparatus at 37° C. With a palladium-charcoal catalyst there was no hydrogen uptake. With Adams catalyst about 0·8 mol. H_2 per Ba atom was absorbed in 4½ hours. The reduced material was found to have retained its activity.

Effect of ketonic reagents.

The antibacterial activity of penicillin disappeared after incubation for 3 hours, at pH 7, with an excess of hydrazine. Incubation with sodium bisulphite for 2 hours at pH 5 also resulted in loss of activity, but this may have been due to the reducing action of the bisulphite, as acid appeared during the reaction.

Inactivation was brought about in 6 hours at room temperature by an excess of hydroxylamine at pH 6. In spite of the buffering action of penicillin at pH 4 it was possible to make an approximate determination of the hydroxylamine absorbed by titrating the acid liberated in the reaction. This corresponded to about 0·8 equivalent per Ba atom.

Attempts to Esterify Penicillin.

Unsuccessful attempts were made to esterify penicillin by the action of various alkyl iodides on its silver salt. This salt was prepared by adding the theoretical amount of a saturated solution of silver sulphate to the Ba salt of penicillin in water, centrifuging the precipitated barium sulphate and drying the supernatant solution from the frozen state. Prepared in this way, it was a very light white powder, easily soluble in water. It was stored in the dark, as decomposition took place on prolonged exposure to daylight. On addition of an excess of methyl iodide, dissolved in benzene or chloroform, to a suspension of the silver salt in the same solvent a violent reaction with instantaneous formation of silver iodide took place. On evaporation of the solvent *in vacuo* at room temperature a yellow oil possessing a characteristic ester-like smell remained. This substance gave a strongly acid reaction and required for neutralization nearly one equivalent of alkali per Ag. atom. This was probably due to rapid saponification of the ester. Similar results were obtained on treating the silver salt with *p*-nitrobenzyliodide and 3 : 5-dibromobenyzliodide. In all three cases silver iodide was formed during the reaction, but the resulting products reacted strongly acid. In attempts to purify the esters by chromatographic adsorption the alcohols corresponding to the iodides used were obtained in pure crystalline state by washing the column with chloroform (or ethyl acetate) and collecting and evaporating the filtrates. The acids remained adsorbed on the top part of the columns and could be eluted with phosphate buffer ; they possessed no antibacterial activity.

DISCUSSION.

The penicillin preparation described in this paper is the most powerful antibacterial agent with predominantly bacteriostatic action so far known. Though it has not yet been obtained crystalline there are indications that it possesses a considerable degree of purity. Thus, on the chromatographic

adsorption column it appears homogeneous. On heating it loses CO_2 corresponding to one molecule per atom of Ba, while less active preparations yield only fractions of a molecule. The nitrogen content corresponds to nearly two atoms per Ba atom ; less active preparations contain less nitrogen, the amount being roughly proportional to the activity. It is not quite certain (E. R. Holiday, see following note) whether the increase in activity obtained by reduction with aluminium amalgam is accompanied by a change in the penicillin molecule, or whether only the impurities are affected.

The small percentage of hydrogen, the large C—Me value and the absence of easily hydrogenated double bonds indicate that the molecule contains several condensed rings ; but the very low extinction coefficient makes the presence of aromatic rings unlikely. There is evidence for the presence of one ketonic, two acetylatable and one latent carboxylic group. If the acidic groups are also assumed to be carboxylic a considerable proportion of the oxygen present is accounted for. With respect to the nitrogen content of the penicillin preparation, it is of interest that few nitrogenous products have been found among the many substances isolated from moulds.

The unusual biological properties of penicillin are linked with an exceptionally unstable chemical configuration. Inactivation by acid, alkali, and by boiling at any pH has been shown to be accompanied by definite chemical changes. Treatment with dilute acid results in the formation of a new titratable group with a pK of approximately 7·6. At the same time two new substances are formed, one with a very characteristic odour, and the other precipitated by base precipitants ; neither of these has yet been identified. Dilute alkali produces a group with a pK of approximately 5·0. Consecutive treatment with acid and alkali, or alkali and acid, does not produce both groups, but only that group resulting from the primary treatment. This suggests that the same part of the molecule is involved in both acid and alkaline inactivation and may be broken down in different ways. The inactivation by boiling is accompanied by the loss of a molecule of CO_2 and other changes involving titratable groups. The nature of the new groups, whether basic or acidic, which are formed in these hydrolytic changes, is under investigation.

SUMMARY.

(1) By means of adsorption, distribution between solvents and reduction a preparation of the barium salt of penicillin was obtained which appeared to be homogeneous by chromatographic analysis. The activity was 450–500 units per mg.

(2) Electrometric titration indicated that the product was the salt of a strong dibasic acid with pK values of approximately 2·3 and 3·5. This, in conjunction with the results of the elementary analysis, suggested a formula such as $C_{24}H_{32}O_{10}N_2Ba$. Evidence was obtained for the presence in the molecule of one carbonyl, one latent carboxylic and two acetylatable groups. At least five C—Me groups were also present, and there was no easily reducible double bond.

(3) Penicillin was very sensitive to oxidizing agents and less sensitive to reducing agents. It was unstable towards dilute acid and alkali, heat, primary

alcohols and various heavy metal ions. Inactivation by acid and alkali was accompanied by the formation of new titratable groups; inactivation by heat also involved a loss of carbon dioxide.

We are indebted to the Medical Research Council and the Rockefeller Foundation for equipment and expenses grants. We wish to express our thanks to D. S. Callow and S. A. Cresswell for valuable assistance in the chemical part of the work, and to R. Callow, M. Lancaster and P. McKegney, under the direction of G. A. Glister, for the production of the crude penicillin.

REFERENCES.

Abraham, E. P., Baker, W., Chain, E., Florey, H. W., Holiday, E. R., and Robinson, R.—(1942) *Nature, Lond.*, **149**, 356.

Abraham, E. P., Chain, E., Fletcher, C. M., Florey, H. W., Gardner, A. D., Heatley, N. G., and Jennings, M. A.—(1941) *Lancet*, ii, 177.

Brandt, L.—(1927) *Chem.-Ztg.*, **51**, 981.

Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., and Sanders, A. G.—(1940) *Lancet*, ii, 226.

Florey, H. W., and Jennings, M. A.—(1942) *Brit. J. exp. Path.*, **23** (following paper).

Freed, M., and Wynne, A. M.—(1936) *Ind. Eng. Chem.* (*Anal.*), **8**, 278.

Miller, B. F., and van Slyke, D. D.—(1936) *J. biol. Chem.*, **114**, 583.

ADDENDUM: THE SPECTROGRAPHIC EXAMINATION OF PENICILLIN PREPARATIONS.

E. R. HOLIDAY.

Crude preparations of the barium salt of penicillin are yellow-orange in colour. As purification proceeds the colour becomes paler. Parallel changes are noted in the absorption spectra. Study of the latter allows division into two types of absorption curve corresponding to—

I.—"Unreduced" pencillin.

II.—"Reduced" penicillin.

Type I curves show high specific extinction coefficients (E) with two distinct maxima at 278–283 mμ. ($E_{0\cdot1\ per\ cent.}$, 14–31) and 375–388 mμ. ($E_{0\cdot1\ per\ cent.}$, 5·0–12·9).

Type II curves show low extinction coefficients with one maximum at shorter wave-lengths (246–254mμ.; $E_{0\cdot1\ per\ cent.}$, 3·6–17·4) and a pronounced long wave inflexion (300 mμ.; $E_{0\cdot1\ per\ cent.}$, 1·4–5·4).

Each batch of penicillin was not systematically followed through all its stages of purification. Most batches were examined at two stages, namely, at the last stage of purification before "reduction" and again immediately after "reduction." One batch was followed through the several chromatographic separations performed on the reduced material. As purification proceeded the type of absorption changed little except at the stage of "reduction," when there was a shift of the maxima to shorter wave-lengths with

reduction in value of E. Preparations, obtained by the " counter-current " extraction with amyl acetate, all showed comparatively low E values (14·0–14·7). After the introduction of the extraction by shaking, E values were higher (21–34·3).

Although the shape and position of the absorption curves of successively purer preparations altered little, the extinction coefficients showed a tendency to become lower. This is illustrated in Fig. 1, in which the ratio Activity/Specific Extinction Coefficient (= A/E) is plotted against activity both for preparations obtained by the counter-current extraction and for those obtained by extraction by the shaking method described above. The lowering of A/E values of preparations obtained by the latter method were either due to the

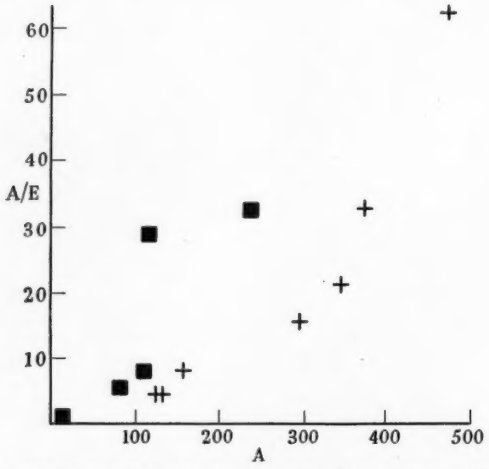


FIG. 1.—Correlation of ratio: Activity (A)/specific extinction coefficient (E) with activity of penicillin preparations.
■ = Preparations by counter-current extraction.
+ = Preparations by shaking extraction.

more efficient extraction of highly absorbing impurities as well as of penicillin, or to the fact that there was coincidentally a change in the metabolic products of the mould. Subsequent chromatographic adsorption made possible the separation of these impurities, with the result that the A/E ratio rose rapidly with increasing purity. It can therefore be said that in successive stages of purification there is a negative correlation between activity and extinction coefficient. In the last stages of purification yielding the preparation of activity 480 Oxford units per mg. visually distinguishable layers of each chromatogram were examined spectrophotometrically. It was hoped that analysis of the different levels of a chromatogram by the well-known method of mixtures together with a knowledge of the activity in each level might give an idea of the state of purity of the preparations. It was found, however, that the number of inactive substances is at least three and that such an

analysis was not feasible. There is therefore nothing in the absorption data to indicate the state of purity of the present purest preparation.

"Reduced" Penicillin.

Absorption measurements indicate that treatment of native penicillin (of activity about 180 units) with aluminium amalgam at pH 6·8 brings about a chemical change in the pigments. The absorption curves of a typical sample before and after reduction are represented in Fig. 2. Considerable purification is brought about by this treatment. This can be accounted for by adsorption on to the aluminium hydroxide of much inactive pigmented material.

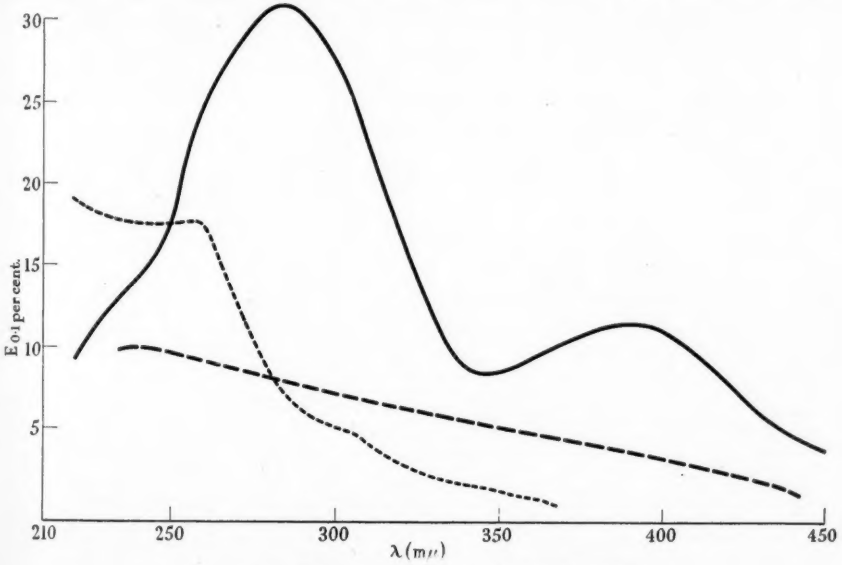


FIG. 2.—Barium salts of native and reduced penicillin.
—— = native; . . . . = reduced; — — — — = eluate.

This inactive pigment when eluted should, if no chemical change has taken place during treatment with the amalgam, show absorption which will account for the difference between the "native" and "reduced" penicillin. Curve 3, Fig. 2, shows the absorption curve of the eluate. No summation of curves 2 and 3, in whatever proportions, can give curve 1. It can therefore be said that most of the material in crude native penicillin preparations is chemically changed by treatment with the amalgam, though it must be emphasized that the portion so changed may not include penicillin itself, since it is not known whether the active substance forms a large or small part of the material at this stage. If it be shown that material of 480 units is almost pure penicillin, then material of 130 units would contain about 30 per cent. penicillin, and it could be stated positively that the penicillin molecule is chemically changed by the

aluminium amalgam treatment without loss of activity. In this connection it has been shown (see main section) that the material of 480 units may undergo catalytic hydrogenation without loss of activity. The change in absorption spectrum from native to " reduced " penicillin is such as would be caused by the reduction of one or more double bonds. With the small amount of chemical evidence as to structure available at present it would be mere speculation to state more than this.

Absorption Spectrum of the Barium Salt of Penicillin of Activity 480 *Units.*

Fig. 3 shows the absorption curve of the penicillin preparation described in the main section, on a basis of g. dry weight/l. The following arguments assume the sample to be almost pure : From the minimum molecular weight

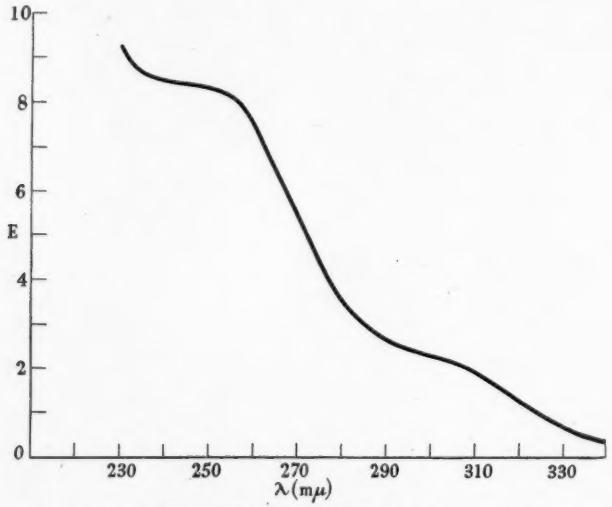
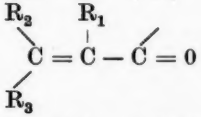


FIG. 3.—Barium salt of penicillin (activity 480 units).
c = 1 g./l. ; d = 1 cm.

of 640 calculated for the barium salt of a dibasic acid having the elementary composition recorded above, the molecular extinction coefficient (E) of the maximum at 247 mμ. is 5420. This is low for any system of substituted aromatic rings such as might be constructed from the empirical formula. It is concluded from this, and the chemical evidence, that the general structure is one of partially or completely saturated poly-substituted hydro-aromatic rings. It is further tempting to apply the generalization brought forward recently by Woodward (1941) and Evans and Gillam (1941) and to postulate a trisubstituted αβ unsaturated ketone group as existing in the molecule. The K and R bonds of Burawoy (1939) would be represented respectively by $E_{max.}$ 247mμ. = 5420 and $E_{max.}$ 300 mμ = 1400. $E_{300} = 1400$ is high for an R bond (chromophore C = O), but taking into consideration the fact that impurities in penicillin preparations show considerable absorption in this

region, further purification may well lead to considerable reduction of absorption at 300 mμ.

The basic structure indicated is—



where R_1 and R_2, R_2 and R_3 may each be cyclically combined or not.

Other absorption data such as the fact that little change is observed in acid or alkali are negative in implication. The inference is that the acidic groups (probably carboxylic) are attached so that the carbonyl group is not conjugated with the main chromophore in the molecule, otherwise one would expect considerable shift of the bands to shorter wave-lengths on passing from neutral solution to strong acid, unless the decarboxylation produced by strong acid prevents the expected effect.

SUMMARY.

1. With increase in purity of penicillin preparations there is a fall in intensity of absorption.

2. The purest preparation (activity 480 units) shows two poorly defined maxima at λ 247 mμ. and λ 300 mμ. $E^{247\ m\mu.}_{0\cdot1\ per\ cent.} = 8\cdot5$ and $E^{300\ m\mu.}_{0\cdot1\ per\ cent.} = 2\cdot3$.

3. There is no evidence from absorption data that the limit of purity has been reached.

4. The activity of penicillin may be destroyed by acid or alkali without altering the absorption spectrum, and the absorption spectrum probably may be altered (by treatment with aluminium amalgam) without altering the activity.

5. Tentative suggestions are made as to structure on the assumption that the barium salt is almost pure, viz.,

(*a*) A polysubstituted hydroaromatic ring structure.

(*b*) The acidic groups (probably carboxyl) not conjugated with the chromophore responsible for the absorption.

(*c*) Possibly the presence of a trisubstituted αβ-unsaturated ketone grouping.

This work was done in the Department of Biochemistry, Oxford. I wish to thank Prof. R. A. Peters for the hospitality and facilities afforded me in his laboratory. I am grateful to Mr. W. Weinstein for making the absorption measurements.

REFERENCES.

Burawoy, M.—(1939) *J. chem. Soc.*, p. 1177.
Evans, L. K., and Gillam, A. E.—(1941) *Ibid.*, p. 815.
Woodward, R. B.—(1941) *J. Amer. chem. Soc.*, **63**, 1123.

SOME BIOLOGICAL PROPERTIES OF HIGHLY PURIFIED PENICILLIN.

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Received for publication March 27, 1942.

As penicillin was further purified it became of interest to re-investigate some of its biological properties. A preceding paper (Abraham and Chain, 1942) gives an account of a purified product containing about 500 units of penicillin activity per mg. The material used on man with some indications of success—the " therapeutic penicillin " of Abraham *et al.*, (1941)—contained 40–50 units per mg., or not more than 10 per cent. of penicillin. There would have been nothing unreasonable in the assumption that further purification would remove inert non-toxic materials, leaving a penicillin product more active against bacteria but more toxic to animal tissues. The following experiments with the purer penicillin of Abraham and Chain (1942) were designed to examine this question.

EXPERIMENTAL.

Antibacterial Activity.

A series of tests were made on sensitive organisms by the dilution method, with the results shown in the Tables I and II. The purest penicillin product so far obtained completely inhibited the growth of staphylococci at a dilution of between 1 in 24 million and 1 in 30 million ; partial inhibition was seen up to 1 in 160 million (Table I).

TABLE I.—*Bacteriostatic Activity of Penicillin Preparations.*

Test organism: *Staph. aureus.*

Stage of purification of the preparation (Abraham and Chain, 1942).	Highest dilution of material (in millions) giving:— Inhibition. Complete.	Partial.
After reduction and before further purification	7	40
Purest material	>24, <30	160

The comparative sensitivity of a number of organisms to a less pure product is shown in Table II. It should be noted that a number of strains of sulphonamide-resistant streptococci (all those which have so far been tested) were sensitive to penicillin. This confirmed an observation made by Colebrook (personal communication) with cruder material. It is also of interest that Salmonella organisms are fairly sensitive.

* With a personal grant from the Medical Research Council.

TABLE II.—*Activity of a Highly Purified Penicillin Preparation against Various Bacteria.*

The preparation used was that obtained after reduction and before further purification (Abraham and Chain, 1942).

Organism.	Strain.	Source.	Highest dilution of material (1 in . . .) giving :— Inhibition.	
			Complete.	Partial.
Staph. aureus	..	Stock culture	7 million	40 million
Strept. pyogenes	A 27	„ „	8 „	16 „
„ „	A (S.R.*)	Infected burn	8 „	16 „
„ „	A 12 (S.R.*)	„ „	8 „	16 „
„ „	Non-haemolytic (1) (S.R.*)	„ „	8 „	16 „
„ „	Non-haemolytic (2) (S.R.*)	„ „	8 „	16 „
Strept. viridans	(1), (2) and (3)	Sputum	8 „	16 „
„ „	(4)	Blood culture	4 „	<8 „
Cl. welchii	..	Stock culture	4 „	<8 „
S. typhi	..	„ „	30,000	120,000
S. gaertneri	..	„ „	30,000	240,000

* S.R. = Sulphonamide resistant (supplied by Col. Colebrook).

Toxicity.

The purest material—that containing 500 units of penicillin activity per mg.—was not available in sufficient quantity for these tests, so the rather less pure was used. Four 20 g. mice were each injected intravenously with 20 mg. of penicillin (sodium salt) in 0·2 c.c. water. One mouse received material containing 250, and three, 325 units per mg. No reaction was observed in any of the mice. The same quantity (20 mg.) of " therapeutic penicillin " (40–50 units per mg.) caused serious embarrassment, though not death (Abraham *et al.*, 1941).

The purer sample of penicillin was thus shown to be less toxic than the impure, and it is reasonable to assume that any further purification will decrease toxicity further—a very satisfactory conclusion when considered in conjunction with the greatly enhanced antibacterial action of the purer preparation.

Action on Leucocytes.

The behaviour of leucocytes was studied by the method previously described (Abraham *et al.*, 1941). The leucocytes were sluggishly motile for an hour in a 1 per cent. solution of penicillin (240 units per mg.) in the saline mixture. This solution was very hypertonic and caused extreme crenation of red cells ; nevertheless the leucocytes were not killed. In a higher dilution (1 in 1600) the leucocytes lived for 3 hours, but there was some variation between individual preparations, and the upper limit at which there was any effect from the penicillin could not be exactly determined. The highly purified material was at any rate not more lethal to leucocytes than the " therapeutic penicillin " of earlier tests.

The Calcium Salt of Penicillin.

Up to the present time the sodium salt of penicillin has been used for therapeutic purposes, but it has the disadvantage of being deliquescent. As the calcium salt, which is not deliquescent, is much easier to handle some trials were made of its toxicity.

Two 20 g. mice each received intravenously 10 mg. of the calcium salt (50 units per mg.) in 0·2 c.c. of water. They became very ill and appeared likely to die, but eventually recovered. Subcutaneous injection of 20 mg. in 0·25 c.c. also caused considerable distress at the time of injection, and three days later the injection area sloughed. No significant reaction was caused by the injection of a 1 per cent. solution into the ear of an albino rabbit.

It was concluded from these experiments that it would be inadvisable to use the calcium salt for therapeutic purposes.

Methods of Assay and Standard of Activity.

At the present time those working on penicillin in different laboratories have adopted no common assay method for determining the biological activity of preparations of penicillin. Some workers prefer to use the dilution method, but it has two disadvantages—that every assay takes a considerable time and that during chemical fractionation the fractions to be tested may become contaminated with bacteria which are not sensitive to penicillin. For routine work, therefore, the ring test is preferred, and the dilution test is kept for occasionally checking the strength of standard or other preparations. For those using the dilution method it may be stated that the " Oxford unit " is that amount of penicillin which when dissolved in 50 ml. of meat extract broth just inhibits completely the growth of the test strain of *Staphylococcus aureus*. Thus material containing one unit of penicillin per mg. just inhibits the growth of *Staphylococcus aureus* at a dilution of 1 in 50,000. Supposing a preparation of penicillin were found to give complete inhibition at 1 in 25,000,000 its activity would be $25{,}000{,}000 \div 50{,}000 = 500$ units per mg.

DISCUSSION.

Purified penicillin has an extremely powerful bacteriostatic action. The material previously used for clinical trials contained not more than 10 per cent. of the active substance. Coincidently with its purification and increased bacteriostatic action the toxicity to animal tissues has fallen. It is clear that up to the present time penicillin is unique in combining great antibacterial activity with absence of toxicity to animal tissues.

It is possible that Salmonella infections will be in the effective therapeutic range of this highly purified material, though much larger doses would be required than against the Gram-positive organisms.

Raistrick and Smith (1941) and Oxford, Raistrick and Smith (1942) have described the antibacterial properties of two other mould products, citrinin and penicillic acid. In the second of these communications they compare the antibacterial activity of pure penicillic acid with that of very impure penicillin; with the further purification of penicillin this comparison becomes invalid.

They quote Alsberg and Black as finding in 1913 that penicillic acid had about the same toxicity as phenol; the toxicity of citrinin has not yet been determined. Without adequate tests no opinion can be formed about the therapeutic possibilities of these substances.

Even if citrinin and penicillic acid should prove to be useful therapeutic agents too much emphasis should not be laid on their easy production and extraction, as there is no doubt that against many organisms penicillin is 500 times as active and is, moreover, certainly non-toxic.

SUMMARY.

The purest preparation of penicillin so far available completely inhibits the growth of *Staph. aureus* at a dilution of between 1 in 24 and 1 in 30 million.

An intravenous injection of 20 mg. of the sodium salt of a somewhat less pure preparation is without apparent effect on a mouse, and human leucocytes survive for an hour in a 1 per cent. solution.

REFERENCES.

ABRAHAM, E. P., AND CHAIN, E.—(1942) *Brit. J. exp. Path.* (preceding paper).

ABRAHAM, E. P., CHAIN, E., FLETCHER, C. M., FLOREY, H. W., GARDNER, A. D., HEATLEY, N. G., AND JENNINGS, M. A.—(1941) *Lancet*, ii, 177.

OXFORD, A. E., RAISTRICK, H., AND SMITH, G.—(1942) *Chemistry and Industry*, **61**, 22.

RAISTRICK, H., AND SMITH, G.—(1941) *Ibid.*, **60**, 828.

PROACTINOMYCIN: A "BACTERIOSTATIC" PRODUCED BY A SPECIES OF PROACTINOMYCES.

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Received for publication March 27, 1942.

DURING the inspection of some agar platings a contaminating colony of a mould attracted attention by the brown discoloration it produced in the medium, and since at the time work on bacterial and fungal antagonisms was in progress, this mould was examined for bacteriostatic action on various micro-organisms. The mould was planted on the centre of plain or enriched agar plates on which *Staphylococcus aureus* or *Streptococcus pyogenes* had previously been spread thinly, and incubated for two days at about 25° C. After further incubation at 37° C. for 24 hours a considerable zone of growth-inhibition of the coccus round the developing fungus was observed. Similar but more extensive zones were obtained if the fungus had previously been grown for two days at 24° C. before the bacteria were added. In both cases the bacteria were spread by flooding a 24-hours broth culture over the plate,

which was then dried by draining off the excess and exposing, with due protection, in the incubator.

EXPERIMENTAL.

The Fungus.

Morphology.

The fungus consists of a branching Gram-positive mycelium like that of an Actinomycete. Aerial hyphae are produced inconstantly on solid media. The only approach to conidia-formation is seen in old cultures, in which many of the hyphae break up into very short cylindrical segments.

Cultivation.

The mould grows richly in 24 hours at 24–25° C. on glucose-agar or serum-agar under aërobic conditions, and, if the inoculation is heavy, a thick, moist, continuous, soft skin of growth is produced which tends to fold and wrinkle from its own pressure. Plain nutrient agar, Sabouraud's agar and other common solid and fluid bacteriological media support growth of varying degrees of richness. On fluid media, including synthetic types, such as those of Czapek-Dox and Kauffmann, the mould grows slowly and in discrete colonies both in the body and on the surface of the medium, and a continuous feltwork is not readily produced. At 37° C. the fungus develops more slowly and it is doubtful whether any " bacteriostatic " is formed.

Biochemical reactions.

Glucose, lactose, maltose, mannitol, saccharose and dulcitol are not fermented. Litmus milk is unchanged in three days, and no indole is formed in peptone water. Gelatin, however, shows liquefaction on the second day.

Pigment.

Since it soon became clear that the " bacteriostatic " and the pigment are different and independent substances, the conditions governing pigment-production were not specially investigated. Pigment is produced in all broth-media (solid or fluid) that have been tested except when glycerin has been added. No pigment is produced in Czapek-Dox synthetic medium.

Identity.

Our provisional view that the mould is an Actinomycete has been confirmed by Dr. Selman A. Waksman of the New Jersey Agricultural Experiment Station, who finds it to possess the characters of his *Proactinomyces* group (Waksman, 1940).

We think that a suitable name for the bacteriostatic substance produced is " Proactinomycin." If other bacteriostatic agents of similar origin should be discovered the agent herein described may be called Proactinomycin 1, the next Proactinomycin 2, and so on. Further, feeling a need for a comprehensive name for bacteriostatic substances, we suggest and have adopted in this

article the use of the word " bacteriostatic " as a substantive, on the analogy of antiseptic, anaesthetic, cosmetic, etc.

The Bacteriostatic Substance.

Production.

In liquid media the mould grows slowly and sparsely, and formation of the " bacteriostatic " is correspondingly weak. Our best method of production was to grow the mould for two days at 25° C. on glucose-agar in rolled Winchester-quart bottles (Epstein and Chain, 1941), and then to add 250 c.c. of glucose-broth to each bottle and shake till most of the mould and agar became detached. The bottles were then left for two more days at 25° C. The fluid thus obtained from batches of 25 Winchesters was mixed and subjected to the extraction process described below.

Extraction and chemical properties.

The anti-bacterial agent has basic properties. It can be extracted from the culture fluid into various organic solvents, such as ether, amyl-acetate, benzene and carbon tetrachloride. From the organic solvents it can be re-extracted into water by adjusting to pH 4 with HCl or H_2SO_4. We have used ether as solvent. The culture fluid is first extracted twice at pH 5 with a quarter of its volume of ether to remove inactive neutral ether-soluble material, and then twice at pH 10 with one-third of its volume of ether to bring out the active material. The ether solution is then extracted twice with one-tenth of its volume of water, the pH being adjusted to 4 with HCl. The aqueous extract is concentrated *in vacuo* and evaporated to dryness from the frozen state. A white powder, very easily soluble in water, is thus obtained. This preparation has been used for all the bacteriological tests. The yield of the material is 60 mg. from 1 l. culture fluid.

The stability of proactinomycin is much greater than that of penicillin. There is only a small loss of activity after boiling for 10 min. at pH 2 or at pH 7. At pH 10 boiling destroys the greater part of the antibacterial activity, whereas incubation at 25° C. for 1½ hours does not affect it appreciably. It is precipitated from aqueous solution by base precipitants such as picric acid, picrolonic acid, flavianic acid, reinecke acid, etc.

Bacteriostatic activity of the preparation.

The growth of various bacteria in broth or serum-broth to which had been added graded quantities of the proactinomycin preparation described above is shown in Table I. It is not implied that purer and therefore more potent preparations may not be obtainable. It is clear that the activity, like that of penicillin, is primarily against Gram-positive organisms and the Neisseriae. Since the tests with streptococci, pneumococci, meningococci and *C. diphtheriae* were all done in 10 per cent. serum-broth it is clear that this amount of serum does not antagonize the bacteriostatic effect. More concentrated serum, pus, etc., have not yet been tried.

TABLE I.—*Bacteriostatic Effect of Proactinomycin.*

Organism.	Approximate highest dilution of material (1 in . . .) giving the effect.
Strept. pneumoniae (Types 13 and 22) . . .	1,500,000
Strept. pyogenes (Group A)	500,000
Staph. aureus	500,000
N. meningitidis (Group 1)	500,000
B. anthracis	500,000
V. cholerae	6,000
S. typhi, S. paratyphi B, Bact. shigae, Bact. coli .	2,000

Toxicity.

This was tested by intravenous injection in mice. It was found that 5 mg. dissolved in 0·1 c.c. of neutral saline solution killed rapidly; 2 mg. was only fatal in a minority of mice, while 1 mg. had no obvious ill effect.* A previous test by intraperitoneal injection had shown 10 mg. to be the lowest certainly fatal dose; 5 mg. was sometimes fatal and always caused toxic symptoms; 2 mg. caused minor transient intoxication, and 1 mg. had no visible effect.

Action on leucocytes.

The preparation (1 in 1000 solution) stopped the movements of leucocytes in a few minutes and killed them within 45 minutes. A 1 in 3000 solution caused death in an hour. At a dilution of 1 in 10,000 the substance caused sluggishness but did not kill within the period of observation, whilst at 1 in 30,000 the leucocytes remained throughout as lively as the controls.†

General.

When compared with penicillin the present material has the great disadvantage of being far more toxic. Further, it would probably be even more difficult to produce in bulk. On the other hand, proactinomycin has the virtue of being more stable and chemically more amenable.

Preliminary Investigation of Other Actinomycetes.

A large number of strains of saprophytic actinomycetes and "proactinomycetes" supplied by the National Collection of Type Cultures were tested for bacteriostatic activity against *Staph. aureus, Strept. pyogenes* and *S. typhi.* Of 52 strains tested, 10 were found to be bacteriostatic in varying degree to the Gram-positive cocci, but none to *S. typhi.* The 10 that showed the effect were (the numbers are those of the N.C.T.C. list; *A* = Actinomyces):

A. californicus, 1561; *A.* 104 (Waksman), 1582; *A. coelicolor*, 2300; *A. reticuli*, 1565; *A. halstedii*, 4980; *Proactinomyces polychromogenes*, 4599; *A. cacaoi* (strain 1), 3558; *A. sampsonii*, 4981; *A. flavus*, 4979; *A. antibioticus* (Waksman), 6124 (Waksman and Woodruff, 1941).

The other 42 strains constitute practically all the remaining saprophytic actinomycetes in the 1936 edition of the N.C.T.C. list.

* These tests were done by L. Berenblum.

† These tests were carried out by M. A. Jennings.

It has long been known that actinomycetes may produce bacteriostatic substances, and Nakhimovskaia (1937) stated that 47 out of 80 species examined showed this property. The subject has been reviewed recently by Waksman (1941). It is evident that this group offers a promising field for further research.

SUMMARY.

The production of a bacteriostatic substance by a species of proactinomyces is described. Its chemical extraction and bacteriostatic activity are discussed, and experiments on its toxicity to mice and leucocytes are recorded.

A note on the bacteriostatic activity of a number of other species of Actinomyces is included.

We are grateful for the highly skilled assistance of Messrs. E. Vincent, D. Callow and A. Haynes; and we are indebted to the Rockefeller Foundation for equipment and grants for expenses.

REFERENCES.

EPSTEIN, L. A., AND CHAIN, E.—(1941) *Brit. J. exp. Path.*, **21**, 339.
NAKHIMOVSKAIA, M. I.—(1937) *Mikrobiologia (U.S.S.R.)*, **6**, 131.
WAKSMAN, S. A.—(1940) *J. Bact.*, **39**, 549.—(1941) *Bact. Rev.*, **5**, 231.
Idem AND WOODRUFF, A. B.—(1941) *J. Bact.*, **42**, 231.

EXPERIMENTS SHOWING THE INFLUENCE OF ONE GROWING TUMOUR UPON ANOTHER.

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Received for publication April 9, 1942.

DURING the last few years our records have shown from time to time that a Jensen's Rat Sarcoma which had stopped growing and was actually going down in size, would sometimes begin to grow again when a graft from a rapidly growing tumour of the same strain was implanted in the opposite flank.

The experiments recorded in this paper were carried out to see how much the rate of growth of one tumour could be influenced by the inoculation of a second graft taken from another tumour growing at a very different rate. The experiments fall into two groups, those in which the second graft was taken from a much faster growing tumour than the original, and those in which the second inoculation was made from a very slow tumour; in every case the same strain of tumour was used.

In the last 30 years many investigators have described the increase in the rate of tumour growth after the inoculation of various kinds of emulsions, many containing extracts of autolysed tumour, but there appear to be few

records showing one growing tumour actually stimulating the growth of another of the same strain. As long ago as 1908, Gierke stated that mice already bearing tumours were better hosts for grafts than normal mice, but his experiments are not entirely convincing as he used a tumour which gave a very variable but always low percentage of takes in normal mice, and compared the growth of a second inoculation into mice which had already been successfully inoculated, with the result of inoculating the same tumour into normal mice.

Russell (1912) showed that a tumour which grows poorly can retard the growth of a rapidly growing tumour of a different strain, and since then numerous authors have noted the inhibiting effect that the disappearance of one tumour or even a small graft has upon another growing in the same animal, either at the same time or afterwards. The inhibiting effect may also be caused by the tumour's disappearance after irradiation or some other treatment, or by the deliberate inoculation of the graft into a situation in which it is known not to grow. Woglom (1929) gives a summary of many of these experiments. The restraining influence on tumour growth shown in the following experiments is not necessarily dependent on the *disappearance* of a tumour, but appears to occur after the inoculation of another tumour growing at a much slower rate than the first.

For many years we have used only one breed of rat, and in over 90 per cent. of these Jensen's Rat Sarcoma grows very quickly. When rapidly growing pieces of tumour are used for grafts, they double their volume in two days or even less (see Fig. 1, Tumour A), and the rats generally have to be killed at the end of three weeks owing to the size of the tumours. When grafts are taken from very slowly growing tumours, they may be in the animal for many weeks or even months.

Stimulation of Tumour Growth after the Inoculation of a Second Graft.

The first group of experiments shows the influence of rapidly growing tumour upon another growing at a slower rate in the same animal.

Experimental Technique.

A number of young rats were chosen of the same sex and approximately the same weight. These were carefully paired so that there was a more or less duplicate rat in each of the two groups. All the rats in both groups were then inoculated with rather slow growing Jensen's Rat Sarcoma. The rats were inoculated in pairs (one from each group), with small pieces of J.R.S. carefully matched under a magnifying glass to ensure that the two groups had grafts of the same size and quality as far as possible.

A few days later one group of the rats was inoculated with small pieces of J.R.S. in the opposite flank to the original graft; these pieces were taken from one of the most rapidly growing tumours of the type already described (see Fig. 1). The interval between the first and second inoculations varied from 5 to 11 days.

After 20 to 28 days' growth, the average volume of the original tumours in those rats which had been given the second inoculation of very rapidly growing J.R.S. was twice as great as the average volume of the control tumours.

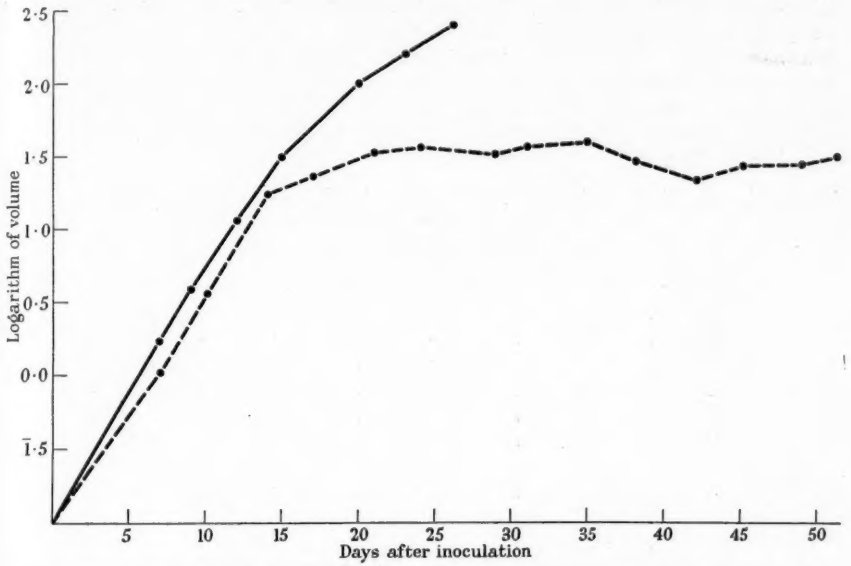

FIG. 1.—Shows the logarithmic rate of growth of two tumours, grafts from which produced contrary effects when inoculated.

——— Quickly growing tumour which stimulated growth (A).
– – – – Slowly growing tumour which impeded growth (B).

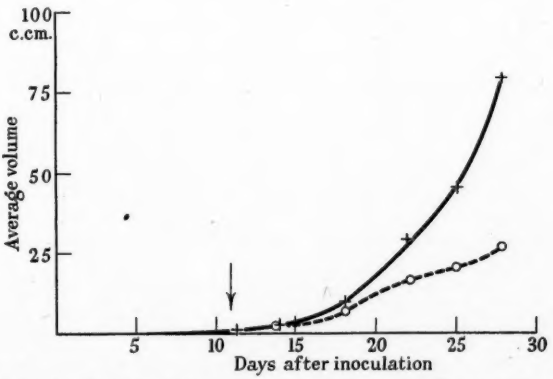

FIG. 2.—Graph showing the stimulating effect produced on tumour growth by the inoculation of a rapidly growing graft on the opposite flank.

——— Average volume of tumours in rats which were given a stimulating inoculation.
– – – Average volume of tumours in control rats.
↓ Marks the day on which second inoculation was given.

A summary of the results of this treatment on ten different occasions, using 198 rats, is given in Table I. The quickening in the rate of growth appeared to begin very soon after the inoculation of the second graft, and became more apparent as time went on. Fig. 2 is a graph of the rate of growth of the original tumours and their controls taken from Experiment 2, Table I, and is a typical example of the stimulation following the inoculation of the quick growing grafts on the opposite flank.

TABLE I.—*Slowly Growing J.R.S. Tumours in Rats which were afterwards given an Inoculation of very Fast Growing Tumour.*

Number of experiment.	Number of rats.	Average volume of original tumours in experimental rats.	Average volume of tumours in control rats.	Time after original inoculation.
1	8	45·1	22·3	25 days
2	16	51·2	20·7	23 ,,
3	16	32·1	10·7	23 ,,
4	10	27·6	23·7	27 ,,
5	16	73·6	26·4	27 ,,
6	34	19·6	15·4	27 ,,
7	24	22·4	9·9	20 ,,
8	14	73·2	41·8	24 ,,
9	36	18·6	7·5	26 ,,
10	24	42·2	30·1	22 ,,
		Average volume per rat.		
	198	30·3	15·8	

The average rate of growth of the control tumours shown in Table I was considerably slower than that of the normal fast tumour. The stimulating effect was most marked in those rats in which the original tumour was growing slowest ; see Experients 3, 7 and 9 in Table I.

Slowing down the Rate of Growth of Tumours by the Inoculation of a Second Graft.

In contrast to the experiments recorded in Table I, tumours growing in rats have been retarded by the inoculation of a second graft taken from a much more slowly growing tumour than the original. The method of inoculation was the same as for the first group of experiments, the only difference being that the second inoculation was made with carefully chosen grafts from a tumour which was either growing very slowly or had begun to go down.

Tumour B in Fig. 1 shows the logarithmic rate of growth of one of the tumours used for the second inoculation.

A total of 52 rats was used for these experiments. Three to four weeks after the original inoculation, the volume of the tumours in the rats which had had a second inoculation with very slow tumour was only ·34 of the volume of the controls. The details are given in Table II and Fig. 3.

In order to produce this marked reduction in the rate of growth, grafts used for the second inoculation must be taken from tumours growing much more slowly than those already in the rats. These experiments need the same care in the choice of tumour inoculated as the first group.

TABLE II.—*Normal J.R.S. Growing in Rats which were afterwards Inoculated with very Slow-growing Tumour.*

Number of experiment.	Number of rats.	Average volume of original tumours in experimental rats.	Average volume of tumours in control rats.	Time after original inoculation.
1	12	16·1	51·8	27 days
2	18	32·2	53·2	23 ,,
3	22	2·4	29·8	21 ,,
		Average volume per rat.		
	52	13·7	42·2	

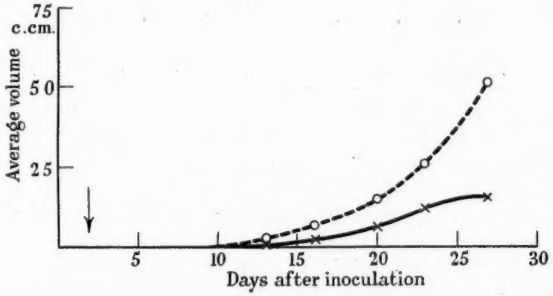

FIG. 3.—Graph showing the effect produced on tumour growth by the inoculation of a graft from a tumour which has stopped growing.

——— Average volume of tumours in rats which were inoculated with a graft taken from a tumour which had stopped growing.

– – – Average volume of tumours in control rats.

↓ Marks the day on which second inoculation was given.

From these results it seemed worth while to find out whether any similar effects could be produced using J.R.S. which had been given a sub-lethal dose of X-rays *in vitro* before inoculation.

Stimulation of X-rayed J.R.S. by Inoculation of Rapidly Growing Normal J.R.S.

Pieces of rapidly growing J.R.S. were given a sub-lethal dose of X-rays *in vitro*; this dose varied from 600*r* to 1000*r* units. Directly the radiation ended two groups of rats were inoculated with carefully matched pieces of the irradiated tumour by the method already described. After some days (4 to 6), all the rats in one group were inoculated on the opposite flank with pieces of very fast growing J.R.S. At the end of approximately a month, the average volume of the X-rayed grafts in the rats which had been given the inoculation of normal tumour on the opposite flank was twice as great as it was in the controls which had had no second inoculation.

A summary of results is given in Table III.

TABLE III.—*X-rayed J.R.S. Growing in Rats which were Afterwards given an Inoculation of Fast-growing Normal J.R.S.*

Number of experiment.	Number of rats.	X-ray dose to tumours before inoculation.	Average volume of X-rayed tumour— in rats which had second inoculation.	Average volume of X-rayed tumour— in rats which had no second inoculation.	Age of tumour.
1	24	720*r* units	58·8	35·9	25 days
2	40	1010*r* ,,	13·9	8·8	26 ,,
3	22	780*r* ,,	10·4	2·5	25 ,,
4	28	600*r* ,,	72·5	51·0	25 ,,
			Average volume per rat.		
	114		38·4	20·6	

A Slowing Down of J.R.S. by Inoculation of X-rayed J.R.S.

Another group of experiments was carried out to see whether tumour tissue which had been given a sub-lethal dose of X-rays (720 to 900*r* units), *in vitro*, would produce the same inhibiting effect when inoculated into rats as did very slowly-growing J.R.S. which had received no irradiation.

Twenty-four rats were treated in this way, the technique being exactly the same as that already described. It was found that when rats, which already had small nodules of normal J.R.S., were inoculated on the opposite flank with pieces of irradiated tumour, the rate of growth of the original tumours in the rats was considerably reduced. At the end of 22 to 24 days, the average volume of the original tumours in the rats which had been inoculated with the irradiated graft on the opposite flank was only about half of the controls. See Table IV.

TABLE IV.—*Normal J.R.S. Growing in Rats which were afterwards Inoculated with X-rayed J.R.S. on Opposite Flank.*

Number of experiment.	Number of rats.	X-ray dose to tumour before inoculation.	Average volume of normal tumour— in rats which had a second inoculation.	Average volume of normal tumour— in rats which had no second inoculation.	Age of tumour.
1	16	900*r* units	34·8	45·1	24 days
2	32	720*r* ,,	31·9	76·5	22 ,,
			Average volume per rat.		
	48		32·8	65·4	

SUMMARY OF RESULTS.

It is possible to speed up the growth of Jensen's Rat Sarcoma growing in one flank of an animal by inoculation of another graft in the opposite flank if the second graft is taken from a tumour growing very much faster than the original graft. An opposite effect can be obtained by inoculating a graft

which will grow very much more slowly than the tumour already growing in the animal.

The tumours in the rats given the stimulating graft in the opposite flank eventually grew to twice the average volume of the controls. The tumours of the rats treated with the slow grafts on the opposite flank were only one-third of the volume of the controls.

Similar stimulation of J.R.S. which had been given a sub-lethal dose of X-rays was shown after the inoculation of a graft of rapidly growing normal tumour. On the other hand the inoculation of a graft of J.R.S. which had been given a sub-lethal dose of X-rays *in vitro* and was growing slowly, had an inhibiting effect on the growth of a normal tumour already growing in the rat, the average volume being only one-half that of the controls.

REFERENCES.

GIERKE, E.—(1908) *3rd Rep. Imp. Cancer Res. Fund*, p. 136.
RUSSELL, B. R. G.—(1912) *5th Rep. Imp. Cancer Res. Fund*, p. 1.
WOGLOM, W. H.—(1929) *Cancer Rev.*, **4**, 130.

THE DETERRENT EFFECT OF LIGHT UPON THE INCIDENCE OF SPONTANEOUS BREAST CANCER IN STRAIN A MICE.

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Received for publication April 22, 1942.

MANY observers have noted the fact that cancer mortality rates vary in different parts of the world, even when peoples of the same European stock, but living in different climates, are compared. In 1941 a comparison of the general cancer mortalities of the various Canadian and American states showed that, in contradistinction to skin cancer, the mortalities fell with (*a*) increasing solar radiation, and (*b*) the number of people exposed thereto (Apperly, 1941). Whether solar radiation is the direct or the indirect cause of the lowered mortality is unknown, but certain experimental work on animals suggests a direct relationship. Thus (*a*) Pierce and Van Allen (1927) and Pierce and Brown (1927) have found in a series of experiments extending over four years that constant light (Mazda and mercury arc lamps) lowers the malignancy (i.e. incidence, mortality and number of metastases) of transplanted cancers in rabbits. (*b*) Morton, Luce-Clausen and Mahoney (1940) found that, of two groups of mice painted with benzpyrene for 17 weeks, the group exposed to artificial daylight for 12 hours daily had fewer tumours (papillomas and skin cancers) than had the group kept in darkness. The appearance of tumours was also delayed. (*c*) Doniach and Mottram (1940) obtained similar results,

but (*d*) Taussig, Cooper and Seelig (1938) using a general electric type SI sun lamp could find little difference between their groups. (*e*) Banzer (1939) reports that both transplantable tumours of rats and spontaneous tumours in mice are diminished in incidence in the tropics as compared with Berlin.

Since nobody, however, has reported similar work on the spontaneous tumours, it was decided to make observations on the effects of light on breast cancer strains of mice. The numbers used, however, were small. These results therefore can be regarded only as a preliminary report, but are published in the hope that others with better facilities than we possess will repeat these experiments on a larger scale.

EXPERIMENTAL.

An attempt to use DBA mice from the Roscoe B. Jackson Memorial Laboratory at Bar Harbour, commenced February, 1937, showed that these mice were completely unsuitable under our laboratory conditions. At 22 months, of those mice which had reached the minimum cancer age of nine months (Little) and had been exposed to light, only two spontaneous breast tumours had developed in the 20 breeding controls (normal rate 65–75 per cent.) and none in the 50 breeding mice. We used a Burdick Cabinet Co. lamp, Type 1A–403, which was found to produce minimum erythema (human in three minutes at 30 in. To this source of light three groups of 25 mice each were exposed for 15, 30 and 45 minutes daily for six days a week, a similar size group being kept as controls. At the minimum breast cancer age, all of the 56 surviving mice, breeding and non-breeding, which had been exposed to light had developed ear or eye tumours. The second and third generations in similar numbers and with similar treatment gave the same general results. Evidently the light dosage was excessive.

A second series of observations was commenced in January, 1939, with 65 strain A mice also from the Roscoe B. Jackson Memorial Laboratory, and a new G. E. Model F ultraviolet lamp producing human erythema at 30 inches in 30 seconds, i.e. one-sixth the light time of our earlier experiments. Because we desired to keep the light below tumour-forming time and intensity we cut the time to one-sixth, or to 2½ minutes daily, at 30 in., giving this amount in divided dosage or for 5 minutes three times a week. No ear tumours developed. The ears first reddened and then tanned slightly, easily distinguishing the light-treated mice from the controls. All animals were kept in metal cages in the diffuse light of a top floor room lighted only by a ground-glass skylight window occupying about one-quarter of the ceiling area. Animals were not exposed singly to the ultra-violet lamp, nor were revolving or circular cages used. Instead, small groups were exposed, in a square cage directly beneath the lamp, the animals being prevented from huddling by means of a stick or by changing the position of the cage at short intervals.

RESULTS.

The results of these experiments are shown in Table I.

TABLE I.

Length of experiment	19 months.
Food : Purina dog chow checkers with a small amount of carrot.	
Minimum tumour age	9 months 4 days.
Number of mice started	65
Number of mice surviving to tumour age	51

(The remaining 14 mice were lost by leukaemia, pneumonia, labour, cannibalism and escape.)

	Controls.	U-V light.
Number of mice reaching tumour age	18	33
Of these :		
(*a*) Mice with one or more litters	15	17
With breast tumours	12	7
Per cent. with tumours	80*	41
(*b*) Mice with no litters	3	16
With breast tumours	1	1
Per cent. with tumours	33	6
(*c*) Ages at which tumours appeared	9 to 17 months	9 to 16 months
Mean	12·5 „	13 „
Average	12·7 „	13 „

* The normal incidence of breast cancer in this strain at Bar Harbour is 80 to 86 per cent.

CONCLUSIONS.

In an attempt to explain the fact that the cancer mortality rates in the various American and Canadian states vary inversely as the solar radiation and the numbers of people exposed thereto, breast cancer strain A mice were exposed to light from a G.E. model F ultraviolet lamp for varying periods. In the small numbers used, the cancer rate in the breeding females was reduced from 80 per cent. to 41 per cent.

Note.—We are grateful to the General Electric Company for the loan of a model F lamp for two years.

REFERENCES.

APPERLY, F. L.—(1941) *Cancer Res.*, **1**, 191.
BANZER, G.—(1939) *Z. Krebsforsch.*, **49**, 443. (Abstract in *Amer. J. Cancer*, 1940, **39**, 262.)
DONIACH, I., AND MOTTRAM, J. C.—(1940) *Amer. J. Cancer*, **39**, 234.
MORTON, J. J., LUCE-CLAUSEN, E. M., AND MAHONEY, E. B.—(1940) *Amer. J. Roentgenol.*, **43**, 896.
PIERCE, L., AND BROWN, W. H.—(1927) *J. exp. Med.*, **45**, 727.
PIERCE, L., AND VAN ALLEN, C. M.—(1927) *Ibid.*, **45**, 483.
TAUSSIG, J., COOPER, Z. K., AND SEELIG, M. G.—(1938) *Surg. Gynec. Obstet.*, **66**, 989.

THE LEUCOCIDIN OF GROUP A HAEMOLYTIC STREPTOCOCCI.

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Received for publication April 24, 1942.

THE usual tests for leucocidin in bacterial filtrates are the Neisser-Wechsberg technique and the microscopic method described by Valentine (1936). The Neisser-Wechsberg method is dependent on decolorization of methylene blue by the reducing action of uninjured leucocytes, and its absence if the leucocytes have been exposed to leucocidin. Streptolysin O (Todd, 1938), which is active only in the presence of reducing agents, cannot be tested for leucocidin by this method since the reducing agent, necessary for activation, causes decolorization of methylene blue. Valentine's method is also unsuitable for this purpose as red blood corpuscles interfere with the action of streptolysin O on leucocytes.

It has been found that normal leucocytes suspended in saline are clumped by NaOH, and that the clumping does not occur if the leucocytes have been previously lysed by bacterial filtrates. This has proved a satisfactory method of testing for leucocidin and it is not affected by reducing agents.

METHOD.

Three or four large mice weighing 35 to 45 g. are inoculated intraperitoneally in the evening with 4·0 c.c. of broth containing 2 per cent. of Difco proteose peptone. The following morning the peritoneal cavities are drained, without applying negative pressure, through large needles (16 British standard wire gauge). The turbid fluid from the peritoneal cavity is collected into tubes containing 2·0 c.c. of 1 per cent. sodium citrate in normal saline. Any tubes which obviously contain red cells are rejected. The leucocytes are centrifuged at slow speed for three minutes and the supernatant fluid is poured off. The leucocytes are washed once in 5·0 c.c. of saline, resuspended in 2·0 c.c. of saline and pooled. This suspension diluted 1 in 10 in saline gives an opacity of about 2 on the barium sulphate scale. A total white cell count is done; and, immediately before using for titrations, the leucocytes are diluted with saline to produce a suspension containing about 5000 white cells per c.mm. A typical differential count is: Polymorphonuclear leucocytes 73 per cent., small lymphocytes 15 per cent., large lymphocytes 4 per cent., undetermined 8 per cent.

Serial dilutions in saline of the fluid to be tested for leucocidin are made in 1·0 c.c. volumes in 3 in. by $\frac{5}{8}$ in. Wassermann tubes. After mixing 0·1 c.c. of leucocyte suspension with each dilution of leucocidin the racks are incubated in a water bath at 37° C. for one hour. The presence or absence of undamaged

leucocytes is then determined by adding 0·1 c.c. of N/1.NaOH containing 2 per cent. of kaolin to each tube and agitating the mixture by striking the bottom of the tube with the finger. If lysis of leucocytes has occurred a uniform suspension of kaolin results, but if undamaged leucocytes are still present they are clumped into threads by the alkali and the clumps are made visible by the kaolin which sticks to the leucocytic agglomeration.

With larger quantities of undamaged leucocytes the addition of N/1 NaOH causes the slow formation of a solid gel so that the tube may be inverted without loss. This, however, is a less satisfactory method of testing for leucocidin, as the large number of leucocytes require correspondingly large doses of leucocidin to cause complete lysis. It is consequently an unsuitable test for weak leucocidins.

Titrations of leucocidins by the soda-kaolin method on a 20 per cent. range of dilutions give sharp end points, and there are usually not more than one or two tubes in which partial lysis is indicated by small aggregations of leucocytes.

After a month's rest the mice are used again for the production of leucocytes. The process can be repeated several times. The yield is usually smallest when normal mice are used for the first time.

STAPHYLOCOCCUS LEUCOCIDIN.

The soda-kaolin method was first tested with staphylococcus leucocidin prepared from strains which were kindly supplied by Dr. F. C. O. Valentine. The soda-kaolin and the microscopic methods give comparable results. In parallel titrations by the two methods the last tube in the series which gives a uniform suspension of kaolin indicates the first tube in the microscopic series in which small numbers of leucocytes begin to appear when stained films of centrifuged deposits are examined. Subsequent tubes which show soda-kaolin clumping have large numbers of leucocytes in stained films.

A rough idea of the titre of staphylococcus leucocidin can be obtained by direct observation of opacity, tubes containing strong leucocidin being less turbid or completely cleared by lysis of leucocytes. This, however, does not apply to streptococcus leucocidin.

STREPTOCOCCUS LEUCOCIDIN.

Streptolysin O (Todd, 1938) has a powerful lytic action on normal mouse leucocytes when tested by the soda-kaolin method in the presence of reducing agents. The ratios of the haemolysin to leucocidin titres of streptolysin O prepared from a number of strains and also the ratios of the neutralizing doses of antistreptolysin O sera on haemolysin and leucocidin indicate that red cells and white cells are probably both lysed by the same substance.

If streptolysin O is prepared in the usual way in glucose-bicarbonate-broth (Todd and Hewitt, 1932), the addition of NaOH in the soda-kaolin test causes a slow precipitation of phosphates which may be confused with the clumping of leucocytes. This is avoided by adjusting broth to pH 8·6, steaming for half an hour, and filtering through Berkefeld candles. Sterile glucose-bicarbonate-phosphate-salt solution is then pipetted into the broth immediately before inoculation with haemolytic streptococci.

Streptolysin O lyses red cells so rapidly that the final results of haemolysin titrations are often completed within 15 minutes. The lysis of white cells is much slower, the final results in this case requiring at least an hour for completion. This leads to two important considerations. In attempts to observe the effect of streptolysin O on leucocytes in mixtures of red and white cells the lysin is rapidly taken up by the red cells before it has had sufficient time to lyse the leucocytes.

Secondly, when titrating the effect of streptolysin O on leucocytes, free from red cells, it is essential to have sufficient reducing agent present in each dilution to prevent inactivation of the lysin by oxidation before the leucocytes are lysed. Leucocidin titrations are therefore done on leucocytes free from red cells with 0·05 per cent. $Na_2S_2O_4$ in each dilution.

Leucocytes are sensitive to acid. Uninoculated broth at pH 6·4 has no definite lytic action on mouse leucocytes in 1 hour at 37° C., but at pH 6·2 there is a definite lytic action which progressively increases with increasing acidity. Leucocidin titrations are therefore done at pH 7·0, although 6·5 is the optimum pH for the action of streptolysin O on red cells (Herbert and Todd, 1941). At pH 8·0 the leucocidin action of streptolysin O is almost entirely abolished.

Each tube in the leucocidin titration first receives 0·5 c.c. of a reducing solution containing 0·1 per cent. of $Na_2S_2O_4$ dissolved in a mixture of equal parts of saline and 2 per cent. peptone-broth at pH 7·0. (Peptone-broth appears to be a better stabilizer than simple buffer solutions.) Dilutions of leucocidin in saline are then pipetted, in 0·5 c.c. volumes, into the tubes; 0·1 c.c. of leucocyte suspension is added and incubation is commenced.

When titrating antileucocidin, dilutions of serum in saline are pipetted into tubes in 0·5 c.c. volumes; a test dose of leucocidin in 0·5 c.c. is then added to each tube, the leucocidin being suitably diluted in a mixture of equal parts of broth and saline at pH 7·0 containing 0·1 per cent. $Na_2S_2O_4$. The mixtures are kept at room temperature for 15 minutes; leucocytes are then added and incubation at 37° C. is commenced.

TABLE I.—*Rapid Lysis of Red Cells and Relatively Slow Lysis of White Cells by Streptolysin O.*

Time of incubation at 37° C.	Volumes of streptolysin O (c.c.).									
	0·5.	0·2.	0·1.	0·05.	0·02.	0·01.	0·005.	0·002.	0·001.	0·00.
Haemolysin titre in 15 minutes	H	H	H	H	H	+++	+	0	0	0
Leucocidin ,, ,,	L	L	L	+	0	0	0	0	0	0
Haemolysin titre in 30 minutes	H	H	H	H	H	+++	++	0	0	0
Leucocidin ,, ,,	L	L	L	L	+	0	0	0	0	0
Haemolysin titre in 1 hour .	H	H	H	H	H	+++	++	+	0	0
Leucocidin ,, ,, .	L	L	L	L	L	+	0	0	0	0
Haemolysin titre in 2 hours	H	H	H	H	H	+++	++	+	0	0
Leucocidin ,, ,, .	L	L	L	L	L	+	0	0	0	0

In all tables H = complete lysis of red cells.
L = complete lysis of leucocytes.
+++, ++, + = degrees of incomplete lysis.
0 = no lysis.

The Time Factor in Haemolysin and Leucocidin Titrations.

Table I shows the difference in time required for the lysis of red cells and of white cells by streptolysin O. Both the titrations were done in the presence of 0·05 per cent. $Na_2S_2O_4$. The volumes used in the haemolysin titrations were 2·0 c.c. of diluted lysin and 0·5 c.c. of 5 per cent. washed rabbit blood cells. The minimal haemolytic dose of 0·02 c.c. completely lysed the test dose of red cells within 15 minutes; on the other hand the same dose of streptolysin O required an hour to lyse the test dose of leucocytes. In these titrations the red cell suspension contained about 740,000 red cells per c.mm. and the white cell suspension about 5200 leucocytes per c.mm. The total numbers of red and white cells lysed were therefore about 370 million and 520 thousand—a ratio of about 700 to 1. Leucocytes are lysed more slowly than red cells and they require a much greater concentration of streptolysin O per cell.

TABLE II.—*The Effect of Red Blood Corpuscles on the Leucocidin Titre of Streptolysin O.*

Action of streptolysin O on—	Testing for haemolysin or leucocidin.	Volumes of streptolysin O (c.c.). 1·5.	1·0.	0·5.	0·2.	0·1.	0·05.	0·02.	0·01.	0·005.	0·002.	0·001.
Red cells alone	Haemolysin	H	H	H	H	H	H	+++	++	+	0	0
White cells alone	Leucocidin	L	L	L	L	L	L	0	0	0	0	0
Mixed red and white cells	Haemolysin	H	H	H	H	H	H	0	0	0	0	0
	Leucocidin	L	0	0	0	0	0	0	0	0	0	0

Prevention of Leucocidin Action by Red Blood Corpuscles.

Table II shows that red blood corpuscles interfere with the lytic action of streptolysin O on leucocytes. The usual doses of rabbits' red blood cells and mouse leucocytes were used for the titrations which were incubated at 37° C. for one hour in a water bath. In the combined titration haemolysis was first observed; soda kaolin was then added and the leucocidin titre was noted. When haemolysin and leucocidin were titrated separately they gave equal titres of 0·05 c.c.; when they were titrated together the haemolysin titre remained unchanged while the leucocidin titre was reduced from 0·05 c.c. to 1·5 c.c. This is an extreme instance with very large numbers of red cells, but it shows the importance of rejecting leucocyte suspensions which contain red cells when titrating leucocidin by the soda-kaolin method.

TABLE III.—*The Effect of Reducing Agents on the Haemolysin and Leucocidin Titres of Streptolysin O.*

Condition of streptolysin O.	Testing for haemolysin or leucocidin.	Volumes of streptolysin O (c.c.). 0·5.	0·2.	0·1.	0·05.	0·02.	0·01.	0·005.	0·002.	0·001.
Fresh filtrate. Not reduced. Diluted in saline	Haemolysin	H	H	H	+++	++	+	0	0	0
	Leucocidin	+	0	0	0	0	0	0	0	0
Reduced. Diluted in saline	Haemolysin	H	H	H	H	H	+++	++	+	0
	Leucocidin	L	L	L	+	0	0	0	0	0
Reduced. Diluted in broth + $Na_2S_2O_4$	Haemolysin	H	H	H	H	H	+++	++	+	0
	Leucocidin	L	L	L	L	L	+	0	0	0

Oxidation of Streptolysin O during Titration of Leucocidin.

Table III demonstrates the necessity of including a reducing agent in each dilution when titrating the leucocidin action of streptolysin O. The fresh filtrate without any reducing agent had practically no leucocidin action although it caused considerable haemolysis. When reduced with 0·1 per cent. $Na_2S_2O_4$ and diluted in saline a weak leucocidin action appeared, but the full leucocidin titre was not attained until $Na_2S_2O_4$ was incorporated in each dilution.

Reduced streptolysin O diluted in saline attained its full haemolysin titre but only 1/5 of its full leucocidin titre. The action of reducing agents in leucocidin titrations is probably to prevent oxidation of the streptolysin O during the period of incubation. This view is supported by the fact that when the times required for complete lysis of the usual test doses of red and white cells were observed it was found that 0·2 c.c. of reduced streptolysin O, diluted in saline, lysed the red cells in two minutes and the white cells in twelve minutes, thus allowing six times longer for oxidation of streptolysin O when testing for leucocidin. This oxidation effect can be imitated in haemolysin titrations by incubating the saline dilutions before adding the red cells, so that time is allowed for oxidation before the very rapid haemolytic action commences.

TABLE IV.—*Lytic Doses of Bacterial Toxins for Leucocytes and for Red Cells. Doses of Neosalvarsan which neutralized the Various Toxins are included for Comparison.*

Bacterial toxin.	Prepared from strain.	Minimal lytic dose in c.c. for— White cells (L).	Red cells (H).	Ratio L/H.	Neutralizing dilutions of neosalvarsan for 2 m.h.d. of lysins.
Streptolysin O	Richards	0·02	0·02	1·0	1/1000
,,	089 M.V. (virulent)	0·05	0·05	1·0	1/1000
,,	089 M.A. (attenuated)	0·03	0·03	1·0	1/1000
,,	Lewis	0·04	0·04	1·0	1/1000
,,	Williamson	0·05	0·05	1·0	1/1000
,,	Cleland	0·06	0·06	1·0	1/1000
Pneumolysin	Pneumococcus	>1·0	0·02	>50·0	1/40
Tetanolysin	T34	>1·0	0·025	>40·0	1/40
Streptolysin S	E14 M.A.*	>0·5	0·01	>50·0	1/2500
Cl. welchii θ toxin	S107†	0·1	0·01	10·0	1/6000
,, α ,,	S107	>0·5	0·05	>10·0	<1/40

* The Weld haemotoxin used in this experiment had the same effect on leucocytes as the normal horse serum used to extract the haemolytic streptococci. In both cases 1·0 c.c. lysed the test dose of leucocytes, while 0·5 c.c. failed to cause complete lysis.

† A dried preparation of *Cl. welchii* θ toxin was kindly supplied by Dr. C. L. Oakley, who found that its combining values were $\theta \equiv 3{\cdot}4$ units. $\alpha \equiv 0{\cdot}03$ units. This had an m.h.d. of 0·001 c.c., the L/H ratio was about 40 and the neutralizing dilution of neosalvarsan was 1/1300.

Ratios of Haemolysin to Leucocidin Titres.

Table IV gives the leucocidin and haemolysin titres of streptolysin O prepared from six different strains of haemolytic streptococci. The table also gives the leucocidin and haemolysin titres of other oxygen-labile haemolysins which are known to be serologically related to streptolysin O, namely pneumolysin and tetanolysin (Todd, 1934) and *Cl. welchii* θ Toxin (Todd, 1941).

Streptolysin S (Todd, 1938) and *Cl. welchii* α toxin, which are not subject to reversible oxidation and reduction and are not serologically related to streptolysin O, are included in the table for comparison because they are derived from organisms which also produce oxygen-labile haemolysins.

The six preparations of streptolysin O had various haemolytic titres, but in each case the titre for haemolysin was the same as the titre for leucocidin. On the other hand the oxygen-labile haemolysins pneumolysin and tetanolysin had no demonstrable action on leucocytes and *Cl. welchii* θ toxin was ten times more effective against red cells than against leucocytes. The oxygen-stable haemolysins streptolysin S and *Cl. welchii* α toxin both failed to lyse leucocytes.

Neutralization by Neosalvarsan.

It has been shown in a previous paper (Todd, 1941) that neosalvarsan neutralizes streptolysin O and *Cl. welchii* θ toxin. The neutralizing doses of neosalvarsan for 2 m.h.d. of the various haemolysins are shown in the last column of Table IV. All the preparations of streptolysin O were neutralized by a dilution of 1 in 1000, pneumolysin and tetanolysin required more neosalvarsan, streptolysin S and *Cl. welchii* θ toxin required less, and *Cl. welchii* α toxin was not neutralized by neosalvarsan in a dilution of 1 in 40.

TABLE V.—*Neutralization of the Haemolysin and Leucocidin Actions of Streptolysin O by Various Sera.*

Nature of serum.	Nature of lysin.	Test dose of lysin. c.c.	Neutralizing dose of serum for leucocidin. c.c.	Neutralizing dose of serum for haemolysin. c.c.	Ratios of neutralizing doses.
Concentrated A.S.O.	S.O. (Richards)	0·08	0·000025	0·000025	1·0
,, ,,	,, (Lewis)	0·16	0·00003	0·00004	1·3
,, ,,	,, (089 M.A.)	0·25	0·00003	0·00004	1·3
,, ,,	Pneumolysin	0·16	—	0·0025	—
,, ,,	Tetanolysin	0·13	—	0·0016	—
Untreated A.S.O.	S.O. (Richards)	0·08	0·0006	0·0008	1·3
,, ,,	,, (Lewis)	0·16	0·0008	0·001	1·3
,, ,,	,, (089 M.A.)	0·25	0·0008	0·001	1·3
,, ,,	Pneumolysin	0·16	—	0·08	—
,, ,,	Tetanolysin	0·13	—	0·004	—
Concentrated θ A.T.	S.O. (Richards)	0·08	0·00016	0·00016	1·0
,, ,,	,, (Lewis)	0·16	0·0002	0·00025	1·3
,, ,,	,, (089 M.A.)	0·25	0·00016	0·0002	1·3
,, ,,	Pneumolysin	0·16	—	0·002	—
,, ,,	Tetanolysin	0·13	—	0·0003	—
Untreated θ A.T.	S.O. (Richards)	0·08	0·0012	0·0016	1·3
,, ,,	,, (Lewis)	0·16	0·0016	0·002	1·3
,, ,,	,, (089 M.A.)	0·25	0·0016	0·002	1·3
,, ,,	Pneumolysin	0·16	—	0·06	—
,, ,,	Tetanolysin	0·13	—	0·002	—

S.O. = streptolysin 0.
A.S.O. = antistreptolysin 0.
θ A.T. = *Cl. welchii* θ antitoxin.
— = insufficient leucocidin action for neutralization test.
Test dose of lysin = 5 m.h.d.

Combining Power of Haemolysin and Leucocidin.

Table V gives the results of titrating four different horse sera against the leucocidin and haemolysin actions of streptolysin O prepared from three

strains of haemolytic streptococci. The sera employed were concentrated and unconcentrated antistreptolysin O and also concentrated and unconcentrated *Cl. welchii θ* antitoxin. It has been shown in a previous paper (Todd, 1941) that *Cl. welchii θ* antitoxin neutralizes the haemolytic action of streptolysin O. This neutralization by an heterologous antibody should supply good evidence as to whether the oxygen-labile haemolysin and leucocidin of haemolytic streptococci are identical substances. The doses of the sera which neutralized haemolysis by pneumolysin and tetanolysin are included in the table for comparison although these substances have no lytic action on leucocytes. In each titration 5 m.h.d. was used as the test dose of lysin.

The titrations were read by noting the smallest dose of serum which completely prevented haemolysis and the smallest dose which prevented any diminution in the size of the leucocyte-kaolin clump. With the quantities used in these titrations, leucocidin and haemolysin should theoretically be neutralized by the same dose of serum if they are a single substance. In practice it was found that a quantity of unneutralized streptolysin O, sufficient to cause a small degree of haemolysis, was usually not sufficient to cause any appreciable diminution in the size of the leucocyte-kaolin clump. It will be seen from Table V that in the majority of cases the ratio of neutralizing doses was 1·3. This means a difference of one tube in the series of dilutions between the antihaemolysin and antileucocidin action of the sera due to the more sensitive method of detecting partially neutralized streptolysin O by small degrees of haemolysis. Nevertheless the ratios of the neutralizing doses of sera for leucocidin and haemolysin support the view that streptolysin O was alone responsible for the destruction of both red and white cells. If the lysis of leucocytes and red cells had been caused by separate though possibly related oxygen-labile lysins, variations in the neutralizing doses of sera similar to those obtained with pneumolysin and tetanolysin for haemolysis might have been expected. In the absence of variations of this magnitude and in view of the evidence set out in the earlier tables it seems to be a fair conclusion that lysis of red and white cells were both caused by streptolysin O.

Relation of Leucocidin to Virulence.

As streptolysin O has a powerful lytic action on mouse leucocytes, an attempt has been made to determine whether there is any correlation between the virulence of a culture for mice and its ability to produce streptolysin O. The method employed was to grow variants of single strains of haemolytic streptococci which had minimal lethal doses of approximately 10^{-6} c.c. and 10^{-1} c.c. in glucose-bicarbonate broth. Filtrates from the cultures were reduced with sodium hydrosulphite and their streptolysin O titres were determined. Under these conditions the variant with attenuated virulence always produced the larger quantity of streptolysin O. An example is given in Table IV of a virulent culture which produced streptolysin O with a minimal lytic dose for both red cells and leucocytes of 0·05 c.c.; the corresponding figure for the attenuated variant of the same strain was 0·03 c.c. This may possibly be due to the different character of growth of the variants. Virulent cultures grow in clumps at the bottom of the tube, while attenuated cultures produce uniform

turbidity so that toxin production is probably more evenly distributed throughout the whole volume. There is no evidence as to which of the variants produces the larger quantity of streptolysin O *in vivo*.

Therapeutic Action of Antileucocidin.

Experiments described in a previous paper (1935) showed that high titred antistreptolysin O sera protect mice to a limited extent against group A haemolytic streptococci of homologous or heterologous type. This protective action is most strikingly evident on the day following inoculation of the infecting dose when the majority of control mice are dead or dying and all the protected mice seem to be perfectly well. On subsequent days a large proportion of the protected mice sicken and die, leaving relatively few survivors at the end of three weeks. The late deaths, which may be delayed for a fortnight or more, often follow an early localization of infection with abscess formation followed later by generalized reinfection from the abscess. It seems reasonable to attribute this protective action of antistreptolysin O to neutralization of leucocidin *in vivo*.

It has previously been shown that antistreptolysin O does not protect mice against haemolytic streptococci unless the protective dose of serum is given at least eight hours before infection. Dochez, Avery and Lancefield (1919) showed that a similar time interval is necessary for protection of mice against group A haemolytic streptococci by antibacterial (anti M) sera. Nevertheless Loewenthal (1939) found combined therapy with antibacterial sera and sulphanilamide to be more effective than either treatment applied separately. It therefore seemed possible that antileucocidin might have a therapeutic action when combined with treatment by sulphanilamide.

A hundred mice weighing 35 to 40 g. were divided into 20 groups. All the mice were inoculated intraperitoneally with doses of haemolytic streptococci (strain L 193, Group A, Type 13) ranging from 10^{-6} c.c. to 10^{-2} c.c. diluted in Ringer's solution so that each dose was contained in 0·5 c.c. Six hours later 25 of the mice which had received the complete range of infecting doses were inoculated subcutaneously with 25,000 units of antistreptolysin O in 0·5 c.c. of concentrated serum ; a second batch of 25 mice were given 20 mg. of sulphanilamide by mouth through a stomach tube ; a third batch were

TABLE VI.—*Combined Therapy with Sulphanilamide and Antileucocidin.*

Dose of culture. c.c.	Number of deaths in each group of five mice.			
	Untreated control mice.	Mice treated with—		
		A.S.O.	Sulphanilamide.	Sulphanilamide + A.S.O.
10^{-6}	1	1	0	0
10^{-5}	3	4	0	0
10^{-4}	4	5	0	1
10^{-3}	5	4	0	3
10^{-2}	5	5	3	3

A.S.O. = concentrated antistreptolysin O horse serum containing 50,000 units per c.c.
10^{-6} c.c. gave about 150 colonies in blood agar.

given combined treatment with the same doses; the fourth batch received no treatment. The strain L 193 was used by Loewenthal (1939) in his experiments on combined therapy. Preliminary experiments showed that it produces a good titre of streptolysin O *in vitro*.

It will be seen from Table VI that, in agreement with previous experiments, antistreptolysin O had no therapeutic action. Sulphanilamide was more effective alone than in combination with antistreptolysin O. This seems to show that although antistreptolysin O has a prophylactic action it has no therapeutic effect on infected mice.

DISCUSSION.

It has been known for many years that leucocytes are lysed *in vivo* when virulent haemolytic streptococci are inoculated into the pleural or peritoneal cavities of animals. Attenuated streptococci do not cause lysis of leucocytes *in vivo*, but this is not necessarily due to absence of potential power to produce leucocidin; it may be due to failure to multiply and invade the tissues of the host—characteristics which are necessary preliminaries to the production *in vivo* of any of the numerous toxins elaborated by haemolytic streptococci. It has been shown in this paper that *in vitro* both virulent and attenuated group A haemolytic streptococci are able to produce streptolysin O, which causes complete lysis of leucocytes when tested at a low oxygen tension in the absence of red blood corpuscles. On the other hand numerous attempts have been made, by growing haemolytic streptococci in a variety of different culture media and by extracting living organisms, to obtain a substance other than streptolysin O which causes lysis of leucocytes *in vitro*. As all such attempts have been unsuccessful there is no evidence of any streptococcal leucocidin apart from streptolysin O, and there is no evidence that this leucocidin is produced in special abundance by virulent organisms.

Channon and McLeod (1929) found that concentrated streptococcal haemolysin lysed leucocytes *in vitro*, and they considered that their haemolysin and leucocidin were probably identical. Gay and Oram (1933) studied streptococcal leucocidin by the methylene blue reduction technique, and came to the conclusion that leucocidin and haemolysin are separate substances. The methylene blue reduction test is, however, an indicator of respiratory failure of leucocytes, and it does not measure their lysis or permanent destruction. Evans (1931) has shown that the acid of culture filtrates prevents the reduction of methylene blue by leucocytes. Uninoculated broth at pH 6·0 will not only interfere with respiration, but will also cause lysis of leucocytes, although the same broth has no lytic effect when the reaction is adjusted to pH 7·0. Weld (1934) found that her haemotoxin, prepared by extracting living haemolytic streptococci with undiluted normal horse serum, caused complete lysis of leucocytes. This may have been due to the action of streptolysin O, since Weld's extracts contain both the streptolysins if they are prepared from strains which produce both streptolysin O and streptolysin S. Although streptolysin O appears to be the only toxin of group A haemolytic streptococci capable of lysing mouse leucocytes, yet the possibility that there are other streptococcal leucocidins at present unknown cannot be definitely excluded.

The experiments described in this paper have been confined to group A strains, and no attempt has been made to prepare leucocidin from strains of haemolytic streptococci belonging to Lancefield's other groups. Human Group C strains and strains belonging to Group G produce substances which appear to be serologically identical with Group A streptolysin O ; it is probable that these substances also act as leucocidins.

It is interesting to note that tetanolysin and pneumolysin have very little action on mouse leucocytes when tested by the soda-kaolin method, although they resemble streptolysin O in their oxygen lability and are neutralized to a considerable extent by antistreptolysin O. On the other hand, *Cl. welchii* θ toxin has a lytic effect on leucocytes which is much less than its haemolytic effect. It is possible that pneumococci behave like staphylococci in producing a leucocidin which is separate and distinct from the haemolysin. No attempt has been made to prepare such a leucocidin from pneumococci.

SUMMARY.

1. A new method is described for titrating leucocidin.

2. Streptolysin O has a powerful lytic action on leucocytes when tested at a low oxygen tension in the absence of red blood corpuscles.

3. Leucocytes are lysed much more slowly than red cells and they require a much greater concentration of streptolysin O per cell.

4. There is no correlation between the production of this leucocidin *in vitro* and virulence.

5. Attempts to prepare a leucocidin other than streptolysin O from Group A haemolytic streptococci have been unsuccessful.

6. Antileucocidin has no therapeutic action for mice and it does not enhance the effect of sulphanilamide in combined treatment.

7. Streptolysin S has no demonstrable action on leucocytes.

8. The oxygen-labile *Cl. welchii* θ toxin has a relatively weak lytic action on leucocytes.

9. Oxygen-labile tetanolysin and pneumolysin have no lytic action on leucocytes.

REFERENCES.

Dochez, A. R., Avery, O. T., and Lancefield, R. C.—(1919) *J. exp. Med.*, **30**, 179.
Evans, A. C.—(1931) *U.S. Pub. Health Rept.*, **46**, 2539.
Herbert, D., and Todd, E. W.—(1941) *Biochem. J.*, **35**, 1124.
Loewenthal, H.—(1939) *Lancet*, i, 197.
Todd, E. W.—(1934) *J. Path. Bact.*, **39**, 299.—(1935) *Ibid.*, **40**, 243.—(1938) *Ibid.*, **47**, 423.—(1941) *Brit. J. exp. Path.*, **22**, 172.
Todd, E. W., and Hewitt, L. F.—(1932) *J. Path. Bact.*, **35**, 973.
Valentine, F. C. O.—(1936) *Lancet*, i, 526.
Weld, J. T.—(1934) *J. exp. Med.*, **59**, 83.

THE VALENCE OF ANTIBODIES AND THE STRUCTURE OF THE ANTIGEN-ANTIBODY PRECIPITATE.

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Received for publication May 13, 1942.

THE important experimental work of Marrack (1938) and of Heidelberger and his co-workers (Heidelberger and Kendall, 1935; Kabat and Heidelberger, 1937) has led these authors to the opinion that the precipitate formed in the precipitin reaction is a network of antigen and antibody molecules. It is assumed that in this network each antigen molecule is bound to several antibody molecules and each antibody molecule to two or more antigen molecules. Hence antibody molecules are considered as *multivalent*; according to Pauling (1940) they are probably *bivalent*, with two active groups on opposite sides of the molecule.

Some of our previous experiments contradict however the obvious assumption of an antigen-antibody network with multivalent antibodies. With arsanil-azo-protein as antigen we had found that the antibody content of the precipitate increases with an increasing arsenic content of the antigen. Arsanil-azo-proteins with 1 to 10 per cent. of arsenic furnished a precipitate, containing 10 to 15 antibody molecules per antigen molecule (Haurowitz, Kraus and Marx, 1936). It is hardly possible to imagine a network with an antibody antigen ratio as high as 50 by 1.

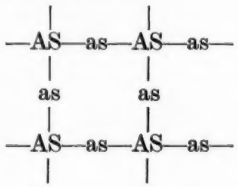
Furthermore one should expect optimal conditions for the formation of a network, if the antigen molecule contained 4 to 10 bonding groups. Actually antigens with less than 10 determinant groups per molecule produced only small precipitates or no precipitate at all.

We had concluded therefore that antibodies are *univalent*, i.e. that they have only one active bonding group per molecule (Haurowitz, 1939) and that the precipitate is formed by complexes of the structure [Antigen.$(\text{Antibody})_N$], where N is lower than the number of determinant groups per antigen molecule but larger than 1·0. The aggregation of these complexes was attributed to the unspecific forces, which bring about the flocculation of euglobulins (Haurowitz, 1939), i.e. to intermolecular heteropolar $-\overset{+}{\text{N}}\text{H}_3 \ldots \overset{-}{\text{O}}\text{OC}$-bridges between globulin surfaces of adjacent particles. For both, precipitation as well as flocculation of euglobulins, are inhibited by higher salt concentrations (Haurowitz, Kraus and Marx, 1936).

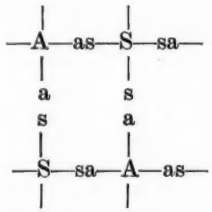
We have examined in the present paper the valence of antibodies by

immunizing rabbits to arsanil-sheep serum globulin, an antigen containing two different determinant factors, the phenyl arsenic acid group and the species specific " Sheep group."

Immunization with such an antigen according to Pauling (1940) should give rise to the formation of three kinds of bivalent antibodies, containing (1) two anti-arsenic groups, or (2) two anti-"sheep " groups, or, finally, (3) both one anti-arsenic and one anti-" Sheep " group. For brevity these three kinds of antibodies may be designated by aa, ss and as, whereas antigens, which carry the relative determinant groups, will be designated by the corresponding capital letters A, S and AS. The as-antibodies should be able to form framework I with the homologous arsanil-sheep serum globulin (AS), and framework II with a mixture of the heterologous arsanil-ovalbumin (A) and sheep serum globulin (S).



I. Precipitation of arsanil-sheep serum globulin (AS).



II. Precipitation of a mixture of arsanil-ovalbumin (A) and sheep serum globulin (S).

We examined this assumption by exhausting an immune serum to arsanil-sheep serum globulin first with arsanil-ovalbumin (A), then with sheep serum globulin (S), and finally with arsanil-sheep serum globulin (AS).

EXPERIMENTAL.

Preparation of antigens.

Sheep serum globulin was precipitated by half saturation of the serum with ammonium sulphate, dissolved in water and reprecipitated ; salts and euglobulin were removed by dialysis. Arsanil proteins were prepared by mixing 6 ml. of a 6 per cent. protein solution with 3 ml. of N./sodium carbonate and a diazo solution, prepared from 0·2 g. of arsanilic acid. The arsanil proteins were reprecipitated by N./HCl and dissolved by the aid of N./NaOH. Their arsenic content was determined by a method described previously (Haurowitz, Kraus and Marx, 1936). Arsanil serum globulin contained 0·87 per cent., arsanil ovalbumin 1·3 per cent of arsenic.

Immunization.

Rabbits were immunized by subcutaneous injection of 2 ml. of a 3 per cent. antigen solution twice a week for 3 weeks. Eight days after the last injection the animals were bled.

Fractional precipitation.

The amount of antigen, indicated in Tables I and II, dissolved in 0·05 to 0·2 ml. of a 0·9 per cent. NaCl solution was admixed to 3 or 5 ml. of antiserum. The mixture was frozen solid and the next day thawed and centrifuged. The supernatant serum was poured as completely as possible in a second cup and the treatment with the same antigen was repeated, until exhaustion was nearly complete. Then the supernatant serum was treated in the same way with the second and third antigen. In Experiment No. 8 the immune serum was treated with a mixture of 0·05 ml. of 3 per cent. arsanil-ovalbumin and the same quantity of sheep serum globulin. All precipitates were washed three times with 2 to 3 ml. of 0·9 per cent. NaCl solution, and once with 50 per cent. methanol, pure methanol, acetone and ether. Then they were dried at 110° and weighed. The antigen content of the precipitates was determined by dissolving them in 1 ml. of 1 per cent. NaOH and measuring the extinction of this solution in the Pulfrich photometer with filter No. S53. 0·1 per cent. solutions of the same antigen in 1 per cent. NaOH were used as standard solutions for photometry.

RESULTS.

Tables I and II show clearly that *antibodies to the arsonic acid group and to the "Sheep" group are precipitated quite independently*; exhaustion with

TABLE I.—*Fractional Precipitation of 5 ml. of an Immune Serum (No. 185) to Arsanil-sheep Serum Globulin.*

Globulin = sheep serum globulin throughout.

Admixed antigen.	Weight of precipitates in mg.				
	1st.	2nd.	3rd.	4th.	Total.
Experiment No. 1:					
2·1 mg. arsanil-ovalbumin	6·34	0·07	..	..	6·41
3·0 mg. globulin	6·53	2·43	1·98	2·06	13·00
4·5 mg. arsanil-globulin	5·66	2·88	1·99	0·60	11·13
					30·54
Experiment No. 2:					
8·4 mg. arsanil-ovalbumin	2·23	0·34	..	..	2·57
3·0 mg. globulin	5·82	2·26	2·53	1·60	12·21
4·5 mg. arsanil-globulin	2·84	1·75	1·47	0·78	6·84
					21·62
Experiment No. 3:					
16·8 mg. arsanil-ovalbumin	1·05	0·12	..	..	1·17
3·0 mg. globulin	6·17	2·30	1·91	1·49	11·87
4·5 mg. arsanil-globulin	2·32	2·07	1·01	0·76	6·16
					19·20
Experiment No. 4:					
3·0 mg. globulin	6·06	2·51	1·88	..	10·45
4·5 mg. arsanil-globulin	12·65	3·75	1·67	..	18·07
					28·52
Experiment No. 5:					
24·0 mg. globulin	13·99	1·64	0·66	..	16·29
4·5 mg. arsanil-globulin	10·75	0·81	0·62	..	12·18
					28·47

TABLE II.—*Fractional Precipitation of 3 ml. of an Immune Serum (No. 199) to Arsanil-sheep Serum Globulin.*

Antigen was admixed in portions of 0·05 ml. of a 3 per cent. solution. The figures of the table show the weight of the precipitates in mg. The weight of the precipitated azoprotein is put in brackets. Globulin = sheep serum globulin throughout.

Admixed antigen.	Weight of precipitates in mg.			
	1st.	2nd.	3rd.	Total.
Experiment No. 6:				
Globulin	3·404	0·420	..	3·824
Arsanil-ovalbumin	7·572 (0·8)	0	..	7·572
Arsanil-globulin	3·928 (1·2)	1·558 (0·65)	..	5·486
				16·882
Experiment No. 7:				
Arsanil-ovalbumin	8·342 (0·9)	tr.	..	8·342
Globulin	2·612	0·774	..	3·386
Arsanil-globulin	4·568 (1·6)	1·714	0·842	7·124
				18·852
Experiment No. 8:				
Arsanil-ovalbumin	..	..	..	..
Globulin	11·334 (0·96)	0·890	..	12·224
Arsanil-globulin	3·682 (1·25)	1·394	..	5·076
				17·300

arsanil ovalbumin did not affect at all the following treatment with sheep serum globulin (Exp. Nos. 1, 2, 3, 4), e.g. 5 ml. of an immune serum after addition of 3 mg. of sheep serum globulin furnished approximately 6 mg. of precipitate, both in the absence of arsanil ovalbumin (Exp. No. 4) and in the presence of a large excess (Exp. No. 3). Furthermore the precipitates obtained with sheep serum globulin after addition of excess arsanil ovalbumin (Exp. Nos. 1, 2, 3 and 7) were quite colourless and free from arsenic. Hence *no common precipitation of arsanil-ovalbumin and sheep serum globulin* had been brought about and no precipitate of the mixed structure II had been formed.

These experiments indicate that *bivalent as-antibodies containing two different bonding groups are absent from immune sera to arsanil-sheep serum globulin.*

The immune sera exhausted by treatment with arsanil-ovalbumin and sheep serum globulin furnished a further precipitate with the homologous arsanil-sheep serum globulin. This precipitation was distinctly inhibited by excess arsanil-ovalbumin (Exp. No. 3), as well as by excess sheep serum globulin (Exp. No. 5). Since the presence of a bivalent (as)-antibody with separate anti-as and anti-" Sheep " groups is excluded by the above mentioned experiments, we attribute the final precipitation of the homologous AS-antigen to a univalent antibody, whose single active group is adjusted to a combined determinant " As-Sheep " group of the antigen.

Our view, according to which antibody molecules are univalent, is supported also by the well-known fact that precipitation is inhibited by an excess of antigen, but never by excess antibody. This different effect of excess antigen

and antibody is particularly striking, when serum globulin is used as antigen; for the physical and chemical properties of antibodies resemble very closely those of serum globulin. The different effect of antigen and antibody excess is best explained by the assumption that antigens are multivalent, antibodies however univalent.

Our experiments do not exclude the existence of bivalent or multivalent antibodies in other antisera, particularly in antisera where no inhibition by excess antigen is observed, e.g. in antisera to haemoglobin (Breinl and Haurowitz, 1936).

SUMMARY.

Fractional precipitation of antisera to arsanil-azo-sheep-serum-globulin leads to the conclusion that such sera contain mainly univalent antibody molecules. Consequently the antigen antibody precipitate is considered as an aggregate of complex particles, each of which consists of a multivalent antigen molecule, to which several univalent antibody molecules are bound.

REFERENCES.

BREINL, F., AND HAUROWITZ, F.—(1930) *Hoppe-Seyl. Z.*, **192**, 45.
HAUROWITZ, F.—(1939) in Kallo's 'Fortschritte der Allergielehre,' Basel (Karger) and New York (Nordman), pp. 53, 59 and 60.
Idem, KRAUS, F., AND MARX, F.—(1936) *Hoppe-Seyl. Z.*, **245**, 23.
HEIDELBERGER, M., AND KENDALL, F. E.—(1935) *J. exp. Med.*, **61**, 563.
KABAT, E. A., AND HEIDELBERGER, M.—(1937) *Ibid.*, **66**, 229.
MARRACK, J. R.—(1938) *Sp. Rep. Ser. med. Res. Counc., Lond.*, No. 230.
PAULING, L.—(1940) *J. Amer. chem. Soc.*, **62**, 2643.